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**Generation of Helicobacter pylori Ghosts by PhiX Protein E-Mediated Inactivation and Their Evaluation as Vaccine Candidates**

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Received 22 July 2002/Returned for modification 24 September 2002/Accepted 15 October 2002

Bacterial ghosts are empty cell envelopes, which may be generated by the controlled expression of the PhiX174 lysis gene E in gram-negative bacteria to obtain vaccine candidates. We describe here the application of this technology to Helicobacter pylori. The lysis gene cassette was cloned into an *Escherichia coli*-Helicobacter pylori shuttle vector and introduced into an *H. pylori* recipient strain by bacterial conjugation. Temperature induction of the lysis gene cassette revealed a quantitative killing of the *H. pylori* culture without induction of lysis-resistant bacteria. Biochemical and transmission electron microscopic studies identified structurally intact *H. pylori*. Prophylactic oral vaccination experiments using these *H. pylori* ghosts in the BALB/c mouse model showed a significant reduction of the bacterial load in the ghost group, as measured by a quantitative bacterial reisolation procedure. Ten of 10 and 5 of 10 mice were protected, respectively, without the use of a mucosal adjuvant. Coadministration of ghosts with cholera toxin as mucosal adjuvant resulted in a complete protection of 10 of 10 and 8 of 8 mice against *H. pylori* challenge, with three animals showing a sterile immunity.

*Helicobacter pylori*, a prevalent gram-negative bacterium, infects half of the world’s population, causing chronic active gastritis, which usually persists throughout life, unless the organism is eradicated (47). Although most infected individuals experience no symptoms, 15 to 20% develop peptic ulcer disease (41). Furthermore, chronic *H. pylori* infection confers a 3- to 12-fold increased risk of developing gastric malignancies, such as adenocarcinoma and low-grade B-cell lymphoma (16, 35, 43).

The use of vaccines for treatment and prevention of *H. pylori* infection has been explored as an alternative to standard multidrug regimens (10). The latter are known to induce antibiotic resistance in *H. pylori* strains (23) and cause the risk of reinfection following eradication (40). Animal studies have shown that immunization with *H. pylori* whole-cell sonicates or purified components is efficient for the prevention of infection, and, more importantly, for the treatment of preexisting infections (5, 8, 12, 14, 28, 31, 32, 50). All successful vaccination protocols included mucosal adjuvants, such as cholera toxin (CT) or *Escherichia coli*-heat-labile toxin (LT), in addition to the antigen. Since CT and LT, and even the genetically detoxified forms of these adjuvants, induce diarrhea in humans (24, 33), it would be desirable to engineer a vaccine without the need of these adjuvants.

One attractive possibility includes the use of recombinant vaccine carrier strains that produce defined *H. pylori* vaccine antigens. Attenuated *Salmonella* vaccine strains (*phoP*/*aroA*) producing the *H. pylori* UreA and UreB subunits can induce protection in mice without the need of a mucosal adjuvant (6, 17). The purpose of the present study was to generate and test a certain form of inactivated bacteria, so-called “bacterial ghosts,” and test them for their potential to induce a prophylactic protection against a challenge with *H. pylori* in the well-established BALB/c mouse model.

Ghosts are empty bacterial cell envelopes without cytoplasm and DNA (46). They are generated by the tightly controlled expression of the cloned lysis gene *E* of bacteriophage PhiX174. The gene *E*-encoded protein was suggested to form a transmembrane tunnel in the bacterial cell wall, through which the cytoplasmic contents are expelled (46). Recent data by Bernhardt et al. (3) suggest that the lysis protein inhibits cell wall synthesis and thus kills the bacteria. Although the mechanism of genetic inactivation is still a matter of debate, the advantage of ghosts is that they share functional and antigenic determinants of the envelope with their living counterparts and thus represent ideal vaccine candidates.

Ghosts have been successfully generated in several gram-negative bacteria, such as *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, *Vibrio cholerae*, *Klebsiella pneumoniae*, and *Actinobacillus pleuropneumoniae* (46). We demonstrate here for the first time the generation of inactivated *H. pylori* ghosts and show that they are able to protect mice against an oral challenge with an infectious dose of *H. pylori*.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** *H. pylori* strains were grown on GC agar plates (Difco) supplemented with horse serum (8%), vancomycin (10 mg/liter), trimethoprim (5 mg/liter), and nystatin (1 mg/liter) (serum plates) and incubated for 24 to 48 h in a microaerophilic atmosphere (85% N2, 10% CO2, 5% O2) at 37°C. *H. pylori* strain P76 was originally obtained from H. Kleanthous, OraVax, Inc., and transformed to streptomycin resistance for optimal quantitation of bacteriophage PhiX174. The gene *E*-encoded protein was suggested to form a transmembrane tunnel in the bacterial cell wall, through which the cytoplasmic contents are expelled (46). Recent data by Bernhardt et al. (3) suggest that the lysis protein inhibits cell wall synthesis and thus kills the bacteria. Although the mechanism of genetic inactivation is still a matter of debate, the advantage of ghosts is that they share functional and antigenic determinants of the envelope with their living counterparts and thus represent ideal vaccine candidates.

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Bertani (LB) agar plates or in LB liquid medium (38) supplemented with chloramphenicol (30 mg/l). Strain 8215S (9) was grown on the same medium supplemented with daiminopimelic acid (0.2 mM).

DNA manipulations. Standard cloning and DNA analysis procedures were performed according to Sambrook et al. (38). Plasmid DNA was purified from E. coli by the boiling procedure, and E. coli cells for electroporation were prepared according to the protocol recommended for the Gene Pulser (Bio-Rad). Plasmid DNA was isolated from H. pylori strains by using Wizard minibeps (Promega) according to the protocol of the manufacturer.

Plasmid construction. Plasmid pHPC38 is the product of subcloning a 2.4-kb DraI fragment of plasmid pAWC10 (unpublished data) into the unique BamHI restriction site of the E. coli-H. pylori shuttle vector pHel2, whereas the BamHI sticky ends of pHel2 have been made blunt by a Klenow fill-in reaction. Plasmid pAWC10, in analogy to plasmid pAWJ (22), carries the E-lysis cassette consisting of gene E, the lambda cI857 repressor gene, and the Apromoter, which has been modified by a single-base-pair exchange to allow repression of the lethal gene E at temperatures up to 38°C.

Natural transformation and bacterial conjugation. Shuttle and suicide plasmids were introduced into H. pylori strains by conjugation or natural transformation as described previously (15). H. pylori transformants or transconjugants carrying the shuttle plasmid pHPC38 were selected on serum plates containing 6 mg of chloramphenicol per liter.

SDS-PAGE and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (26) with a mini-slab apparatus. Proteins separated by SDS-PAGE were transferred to nitrocellulose membranes in a semidy blot apparatus at a current density of 0.01 mA/cm². Unreacted sites of the nitrocellulose membrane were blocked with a 3% (wt/vol) solution of bovine serum albumin (BSA) in TBS (20 mM Tris-HCl, pH 7.5, 150 mM NaCl). The nitrocellulose membrane was then incubated with an appropriate dilution of antibody for 2 h and washed three times with TBS containing 0.5% (vol/vol) Tween 20. Subsequently, alkaline phosphatase conjugated to protein A was added to TBS containing 3% (wt/vol) BSA. After incubation for 1 h, the nitrocellulose membrane was washed three times with TBS containing 0.5% (vol/vol) Tween 20 and developed with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium.

Electron microscopic analysis. For negative staining, thin carbon support films were prepared by indirect sublimation of carbon on to freshly cleaved mica. Samples were then absorbed to the carbon film and negatively stained with 1% (wt/vol) aqueous uranyl acetate (pH 4.5), as described previously (49). After air drying, samples were examined by transmission electron microscopy (TEM) in a Zeiss TEM 910 at an acceleration voltage of 80 kV. For embedding, after fixation of samples in 1% formaldehyde, samples were dehydrated in a graded series of acetone and embedded with Spurr epoxy resin according to the described protocol (42). Ultrathin sections were counterstained with uranyl acetate and lead citrate before examination in a Zeiss TEM 910.

Animals. Age-matched (6 to 8 weeks) female BALB/c mice were obtained from RCC, Itingen, Switzerland. All protocols involving animal experimentation were approved by the Regierung von Oberbayern (Aktenzeichen 211-2531-60/98).

Growth of H. pylori for oral infection of mice. For infection of mice, strain P79 was grown for 2 days on serum plates/strat at 37°C, harvested, and suspended in Brucella broth (Oxoid, Ltd., Basingstoke, England), and the final concentration was adjusted to 3.5 × 10⁷ cells per ml. Mice were inoculated three times intragastrically at 2-day intervals with 0.3 ml of bacterial suspension (10⁵ bacteria).

Preparation of H. pylori ghosts. H. pylori P79 (pHPC38) was grown on Brucella broth agar plates containing 10% horse serum and chloramphenicol (10 μg/ml). After a 2-day incubation at 36°C under a microaerobic atmosphere, cells were harvested in 10 ml of brain heart infusion (BHI) supplemented with 10% fetal calf serum and chloramphenicol (10 μg/ml) and grown for another 5 h under mild agitation (90 rpm). Before the culture reached the late-logarithmic growth phase, 10 to 50 ml of fresh medium was added. By repeating the addition of increasing amounts of fresh medium, the culture thus was expanded to 3.5 liters. When the growing culture reached an optical density at 600 nm (OD₆₀₀) of 0.5, the incubation temperature was shifted up to 42°C to induce the gene E-lysis lysis process. After another 15 h of incubation at 42°C with monitoring of the OD₆₀₀, the lysed culture was harvested. A centrifugation step (4°C, 10,000 g, 10 min) served to pellet the ghosts, which were then resuspended in 100 ml of ice-cold phosphate-buffered saline (PBS). After the centrifugation and resuspension had been repeated twice more, the numbers of ghosts were determined under the microscope with a Thoma counting chamber, and aliquots of 2.5 × 10⁸ ghosts per 100 μl were stored at −70°C until use.

Infection and reisolation of bacteria and quantitative culture. At the endpoint of the experiment, mice were anesthetized by CO₂ and sacrificed by cervical dislocation. The stomach was removed, weighed, and opened along the great curvature. For assessment of H. pylori colonization by quantitative culture, weighed stomachs were homogenized in 2 ml of Brucella broth by a hand homogenizer (Fisher Scientific, Germany), and serial dilutions of 1 in 20 were spread over the surface of serum plates/strep (performed in duplicate). The plates were incubated for 5 days, and colonies were counted to determine the number of CFU per gram of stomach tissue.

Statistical analysis. In the vaccination experiments, statistical significance between the groups was determined by the nonparametric Wilcoxon signed-rank (two-tailed) test. P values of <0.05 are considered as significantly different.

RESULTS

Construction of a plasmid-based PhiX174 gene E lysis cassette for generation of H. pylori ghosts. The E. coli-H. pylori shuttle plasmid pHel2 was used as the basis for the construction of an H. pylori lysis plasmid. This vector allows stable replication in both E. coli and H. pylori (20). The PhiX174 lysis gene cassette consists of the PhiX174 lysis gene cassette was ligated into the BamHI site of the multiple cloning site of shuttle plasmid pHel2 and transformed into E. coli DH5α. At temperatures below 38°C, re-
At time zero, the cultures were shifted from 28°C to 42°C. leads to a recovery of the culture DH5α(P79(pHPC38)). Prolonged incubation at 42°C (up to 24 h) leads to a recovery of the culture DH5α(P79(pHPC38)) due to the emergence of lysis-resistant bacteria. (B) The H. pylori culture was grown at 35°C and shifted at time zero to 42°C. H. pylori P79(pHPC38); P79(pHel2). Prolonged incubation at 42°C (up to 48 h) does not lead to recovery of the culture P79(pHPC38).

Combinant plasmids could be isolated. One such plasmid, characterized by restriction analysis and designated as pHPC38, was used for all further experiments (Fig. 1).

Testing of the lysis gene cassette in E. coli by temperature induction. The functional expression of gene E from plasmid pHPC38 was tested in E. coli DH5α by growing the bacteria in LB medium and monitoring their growth by measuring the ODs of the culture at various time points (OD550). As a control, DH5α(pHel2) was used. The cultures were shifted from 28°C to 42°C at 0 h, which resulted in the functional inactivation of the cI857 repressor and transcription of E by the H. pylori promoter. After induction at 42°C, the OD of DH5α[pHPC38] increased to an OD550 of 1.25 within 1 h, but dropped rapidly to 0.6 (Fig. 2A). Strain DH5α carrying the cloning vector pHel2 alone showed a strong increase in its OD at the same time interval (OD550 = 1.9). DH5α(pHPC38) grown at the permissive temperature of <38°C showed growth characteristics similar to those of the control strain DH5α(pHel2) without lysis gene expression (data not shown). In E. coli, which is the natural host of bacteriophage PhiX174, mutant lysis-resistant bacteria had already appeared several hours after induction (data not shown). The experiment demonstrated both that the lysis gene cassette cloned in the E. coli-H. pylori shuttle vector was induced under the elevated temperature and that the system was functional in E. coli DH5α.

Transfer of PhiX174 gene E into H. pylori by natural transformation or conjugation. To test whether H. pylori was also sensitive to the PhiX174 gene E-mediated lysis procedure, we attempted to introduce the plasmid pHPC38 into H. pylori strains by natural transformation as well as by bacterial conjugation. Although different H. pylori strains were employed as recipients, natural transformation of the plasmid did not result in any transformants with plasmid pHPC38. With the E. coli host strain β2155 as a donor and H. pylori strain P79 as a recipient, bacterial conjugation resulted in H. pylori transconjugants after selection at 35°C. The plasmid was isolated from P79(pHPC38), and restriction digests revealed a stable replication of the plasmid at the permissive temperature (35°C) on serum plates.

To induce the temperature-dependent expression system, the recombinant strain P79[pHPC38] was grown in Brucella medium at 35°C to an OD550 of 0.35. The culture was then shifted to 42°C by gentle shaking (90 rpm). After temperature induction, the OD of the culture increased continuously for 3 h, a slight reduction in the OD550 was visible 4 h after induction, and the minimal OD was measured 9 h after induction (OD550 = 0.25) (Fig. 2B). Interestingly, the OD of the culture did not increase any further, even after prolonged incubation of up to 48 h (Fig. 2B). In contrast, H. pylori P79(pHel2) carrying the empty cloning vector was not hampered in its growth behavior at 42°C for up to 28 h and showed only a slight reduction after 48 h (Fig. 2B).

Analysis of the viability of the induced cultures by plating on serum plates revealed colony formation and growth of the P79(pHel2) control strain at the elevated temperature after 48 h, but no growth of P79(pHPC38) was observed (data not shown), indicating that no lysis-resistant H. pylori mutants arose and that the H. pylori culture was quantitatively inactivated. The generation of nonculturable coccoid mutants of P79(pHPC38) following temperature induction could be excluded by phase-contrast microscopic inspection of the culture. In the P79(pHel2) control, coccoid forms were identified that probably resulted in the reduction of the OD550 of the culture and a slight reduction in the CFU after 48 h at 42°C. The fluorescence in situ hybridization (FISH) technique, developed recently in our laboratory, detects both vegetative and coccoid forms of H. pylori very efficiently (48), but interestingly, FISH did not detect protein E-inactivated H. pylori P79, indicating that tRNA was not accessible by FISH or was absent in the inactivated bacteria (data not shown).

Characterization of the inactivated H. pylori ghosts by TEM.
Next, TEM was used to compare P79 and P79(pHPC38), both grown at 42°C for 48 h (Fig. 3). By viewing ultrathin sections (Fig. 3A and C) or negative-stained specimens (Fig. 3E), *H. pylori* P79 showed no deviation from its vegetative form, and membranes appeared to be intact, indicating that the temperature stress did not have a deleterious effect on the *H. pylori* strain. The situation was completely different for P79(pHPC38). Analysis of ultrathin sections revealed a partial disintegration of the vegetative form, probably due to the strong disruption of the cytoplasmic membrane and the cell wall (Fig. 3B and D). The outer membrane looked mostly intact, but was sometimes found to be detached from the bacterial cytoskeleton (Fig. 3F, negative stain). The cytoplasmic content apparently did not leak out completely.

To obtain further data on the protein level of P79(pHPC38), the genetically inactivated *H. pylori* cells were analyzed by Western blotting with specific antisera directed against proteins of the outer membrane and the cytoplasm (Table 1).
May be from the proposed phage inactivation approach. Several genes, such as recA (39), ureA, and ureB (25), and pfr (2) could be identified in the lysate of the inactivated bacteria with the corresponding specific antibodies. A significant reduction of these cytoplasmic proteins compared to the level of typical outer membrane proteins such as alpA (37) or BabA (21) was not observed, although the Western blot procedure did not provide quantitative data.

Taken together, our data indicated that H. pylori P79 was efficiently inactivated by the PhiX-mediated lysis gene expression system, but the cytoplasmic content of the bacteria was not completely expelled, as described for other ghosts induced by the PhiX-mediated expression system (46).

**Evaluation of ghosts for prophylactic vaccination in the H. pylori mouse model.** Groups of 10 BALB/c mice were orally immunized with three doses of $2.5 \times 10^9$ ghosts or ghosts plus CT (10 μg per mouse) as mucosal adjuvant on days 0, 7, and 14. Control mice received PBS (infected group) or no treatment at all (naïve group). Three weeks after the last immunization, all groups of mice except the naïve group were challenged with a streptomycin-resistant, mouse-adapted H. pylori strain, P76 ($10^9$ CFU, three times with 2-day intervals), and 4 weeks later, the animals were sacrificed and gastric colonization was assessed by quantitative culture of P76 on serum plates containing streptomycin.

As compared to the naïve group (Fig. 4A), the infection control (PBS group, Fig. 4A) shows significant infection, with a median of $1.85 \times 10^6$ CFU of P76 per g of stomach tissue. The ghost group (Fig. 4A) with a median of $6.75 \times 10^5$ is significantly reduced, compared to that in the infection control (~96%). Only a slightly stronger reduction in colonization was found with the ghosts used together with CT as adjuvant (Fig. 4A), which resulted in a value of $3.78 \times 10^5$ as the median of infection (~98%). The reduction in CFU over 2 logs indicates a protection of all animals in the ghost group, as well as the ghost plus CT group.

In an attempt to reproduce and validate the data from the first experiment, a second experiment with a novel batch of bacterial ghosts was performed with the same vaccination strategy. Again, the animals vaccinated with ghosts plus CT showed the highest reduction rate in bacterial load (Fig. 4B), followed by the ghost group (Fig. 4B). The total reduction rate in the ghost group was less efficient than that in the first experiment, but was still significant. Five of 10 mice had enough of a reduction in the amount of CFU per gram of stomach to classify them as protected, with 1 animal showing a sterile immunity.

**DISCUSSION**

A major problem for development of an efficient vaccine against H. pylori in humans seems to be the choice of an appropriate mucosal adjuvant (44). Due to intestinal toxicity, the dose of heat-labile toxin had to be reduced from 10 to 5 μg in a phase I-II clinical trial, where recombinant H. pylori urease was given orally to H. pylori-infected human volunteers (33).

In the H. pylori mouse model, the use of recombinant vaccine carrier strains, such as attenuated Salmonella strains producing the ureA and ureB proteins, revealed protection in a prophylactic immunization experiment without the use of a mucosal adjuvant (6, 17). Although this approach has several
advantages, one limitation might be that only a restricted number of antigens of the vaccine strain can be produced in the carrier strain. In recent safety and immunogenicity studies of pohP/phqO-deleted Salmonella enterica serovar Typhi (11) or strain Ty21a (4) expressing H. pylori urease in adult volunteers, the vaccination was found to be safe. Although volunteers mounted an immune response against the Salmonella carrier antigens, no volunteer had detectable mucosal immune responses to the urease antigen (4, 11), but three of nine volunteers showed a weak but significant T-cell response to H. pylori urease (4).

Our study was aimed to test a further concept of antigen delivery for oral vaccination, the bacterial ghosts, which present a large number of bacterial antigens to the immune system. We hypothesized that this approach might avoid the use of adjuvant due to the rather acid- and protease-resistant nature of the bacterial ghost antigens and the presence of cell wall components with adjuvant properties. These characteristics are not found in soluble purified antigens or bacterial lysates. Thus, bacterial ghosts present a complex combination of structurally intact, rather than single, defined bacterial antigens—such as in the recombinant attenuated Salmonella approach—to the immune system.

By definition, bacterial ghosts are empty cell envelopes of gram-negative bacteria, which might be generated by expression of the bacteriophage PhiX gene E in the bacteria of choice (51). Genetic inactivation of the microorganisms leaves an intact bacterial surface. Chemical or thermal inactivation procedures may lead to the destruction of immunogenic epitopes, which might cause reduced immunogenicity (13, 34). We adapted the method here to H. pylori by expressing the E gene from a shuttle plasmid in H. pylori under the control of the $\lambda P_{\text{rmaa}}$ temperature-inducible promoter. We find a quantitative inactivation of the H. pylori culture, without induction of lysis-resistant bacteria. The lack of lysis-resistant mutants in H. pylori might be due to the fact that H. pylori is not a natural host of the phage PhiX, but the mechanism of this genetic inactivation in H. pylori is not understood.

Although the negative-staining electron microscopy data indicate that the cytoplasmic membrane is disrupted (Fig. 3), the disruption seems not to be efficient enough that the cytoplasmic content leaks out completely. This could be a problem with the strength of gene E expression in H. pylori, which might be reduced in comparison to that in E. coli or other bacteria. Since we do not have an antiserum against the PhiX protein we were unable to test the expression or the putative localization of the protein in H. pylori directly. To our knowledge the $\lambda P_{\text{rmaa}}$ promoter has not been used in H. pylori before, and nothing is known about its strength. The generation of completely empty H. pylori ghosts is still a further aim in our laboratory. This might be attained by stronger expression of the gene E or by coexpression of a DNase in H. pylori, which would degrade the viscous bacterial DNA to allow a complete emptying of the ghosts.

We used here for the first time H. pylori ghosts for vaccination experiments in the H. pylori mouse model. Although several gram-negative bacteria have been used successfully to generate bacterial ghosts, only a limited number of vaccination experiments have been performed with this type of antigen presentation. When pigs were vaccinated intramuscularly with a dose of $5 \times 10^9$ CFU of A. pleuropneumoniae ghosts or formalin-inactivated bacteria, colonization of the respiratory tract after challenge with A. pleuropneumoniae was only prevented in the ghost-vaccinated group (19).

In our experiments, we observed a clear reduction of the bacterial load in both independent vaccination experiments compared to the load in the PBS-vaccinated control (Fig. 4). A reduction of approximately 1.5 to 2 logs is considered protective vaccination, since sterile immunity is usually not obtained in the H. pylori mouse vaccination models (45). It has been shown in several studies in Helicobacter mouse models that without coadministration of a mucosal adjuvant, such as CT, LT, or mutant LT K63, to purified H. pylori proteins or whole-cell sonicates, no protective response was observed (7, 27, 29, 30). In our first experiment with H. pylori ghosts for vaccination, we obtained a reduction in the H. pylori bacterial load that is in the same range or even better than that reported for vaccination experiments with recombinant Salmonella producing H. pylori urease (1) or H. pylori sonicate plus CT (45). If we coadminister H. pylori ghosts with CT, the colonization rate can be further reduced, culminating in a sterile immunity in 3 out of 18 animals (Fig. 4).

The second vaccination experiment resulted in clearly less efficient protection in the ghost group. Since the procedure was the same as for the first experiment and the positive and negative controls (sham infected and ghosts plus CT) were comparable to those in the first experiment, it might be that the quality of the ghosts was not as high as that for the first experiment. This would indicate that the expression system has to be optimized for H. pylori, and the lysis procedure has to be controlled more rigorously to ensure that a consistent form of ghosts is produced.

In our experiments, we employed only a fixed time window for challenge and reisolation of the bacteria, and a high dose of antigen was used. As demonstrated recently by Sutton et al. (45), the dose of antigen and the time points of vaccination are important parameters that have a consequence on the vaccination result. Thus, our initial data indicate that H. pylori ghosts have to be considered as an interesting alternative to the vaccination techniques used so far to control the H. pylori infection. In future experiments, it will be necessary to optimize the vaccination parameters carefully for H. pylori ghosts. By optimizing gene expression of the E gene in H. pylori and by improving the treatment conditions after lysis, it might be feasible to generate completely empty ghosts of H. pylori. Furthermore, it will be necessary to examine the possible induction of postimmunization gastritis in vaccinated animals, as well as look for key parameters of humoral and cellular immune response elicited by the ghost vaccine.

ACKNOWLEDGMENTS

We thank B. P. Burns for critical reading of the manuscript. We are grateful for the receipt of the mouse-adapted H. pylori strain by H. Kleanthous, OraVax, Inc.

This work was supported by EVAX Technologies, Munich, Germany, and the Deutsche Forschungsgemeinschaft (grant HA 2697/4-1 to R.H.).

REFERENCES

1. Aeberscher, T., S. Laforsch, R. Hurwitz, F. Brombacher, and T. F. Meyer. 2001. Immunity against Helicobacter pylori: significance of interleukin-4 re-
