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In Vivo Complementation of *ureB* Restores the Ability of *Helicobacter pylori* To Colonize

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The objective of this study was to determine (i) if complementation of *ureB*-negative *Helicobacter pylori* restores colonization and (ii) if urease is a useful reporter for promoter activity in vivo. Strains used were M6, M6Δ*ureB*, and 10 recombinant derivatives of M6 or M6Δ*ureB* in which urease expression was under the control of different *H. pylori* promoters. Mice were orally inoculated with either the wild type or one of the mutant strains, and colonization, in vivo urease activity, and extent of gastritis were determined. Of eight M6Δ*ureB* recombinants tested, four colonized mice. Of those, three had the highest in vitro urease activity of any of the recombinants, significantly different from that of the noncolonizing mutants. The fourth colonizing recombinant, with *ureB* under control of the *cag-15* promoter, had in vitro urease activity which did not differ significantly from the noncolonizing strains. In vivo, urease activities of the four colonizing transformants and the wild-type control were indistinguishable. There were no differences in gastritis or epithelial lesions between mice infected with M6 and those infected with the transformants. These results demonstrate that recovery of urease activity can restore colonizing ability to urease-negative *H. pylori*. They also suggest that *cag-15* is upregulated in vivo, as was previously suggested by demonstrating that it is upregulated upon contact with epithelial cells. Finally, our results suggest that total urease activity and colonization density do not contribute to gastritis due to *H. pylori*.

The link between *Helicobacter pylori* and peptic ulcer, first recognized by Barry Marshall and Robin Warren in 1982 (23, 24), provided major insight into human gastric pathology. In its most severe manifestations *H. pylori* can be responsible for gastric disease ranging from peptic ulcer to gastric cancer (24, 29, 30). Infection with *H. pylori* is common worldwide. Colonization ranges from 50 to 100%, making *H. pylori* the most common infectious agent of humans in the world today.

Because of the prevalence and importance of *H. pylori* infection, understanding the mechanisms by which it colonizes the gastric mucosa and causes disease has received intense interest. *H. pylori* possesses several putative colonization factors, including urease (23, 24), various adhesins (13, 14, 28, 35), and flagellar motility (19, 23), some of which have been shown to be necessary for gastric colonization (1, 2, 5, 11, 36). Of these factors, urease was the first described and is probably most widely studied. Strong urease activity was noted in the initial description of *H. pylori* (23), and subsequent studies have implicated urease activity as an important virulence factor. Urease accounts for about 5% of *H. pylori* protein (18) and is consistently present in all naturally occurring strains. Further, genetically engineered urease-deficient *H. pylori* is unable to colonize either germfree piglets (5), ferrets (2), or mice (36). It is thought that urease may contribute to gastric damage due to *H. pylori* physical injury from ammonia (4, 21, 26), inflammation due to a host immune response to the protein (12, 16), or other means of mucosal damage (33, 34). Taken together, these data indicate an important role for urease both for promoting colonization and in the pathogenesis of gastric disease.

Urease is a nickel metalloenzyme that catalyzes the hydrolysis of urea to ammonia and carbon dioxide. Synthesis of active urease by *H. pylori* requires the presence of the structural genes *ureA* and *ureB*, which associate to form the 550-kDa holoenzyme (18, 22), and the accessory genes, *ureIEFGH*, which are necessary for full expression of urease activity (3). In addition, nickel transport enzymes such as NixA (27) are required for full expression of urease activity. Animal experiments with urease deletion mutants have demonstrated that *ureB*, *ureG*, and *ureI* are all necessary for gastric colonization (2, 5, 32, 36).

One limitation of *H. pylori* genetic deletion studies is that, until recently, in vivo genetic complementation has not been possible. Shuttle plasmids that allow expression of genes in *H. pylori* have been described (17), but these tend to be strain specific and difficult or impossible to maintain in animal-virulent *H. pylori* strains (unpublished observations). Thus, evaluation of the role of colonization and virulence factors has depended on experiments demonstrating loss of virulence due to loss of the factor to be tested. Here we show that a transcriptional reporter system which utilizes urease production as a measure of gene expression (20) complements a urease-negative, *ureB*-null mutant strain and restores both urease activity and the ability to colonize mice. This confirms the essential nature of urease activity for colonization and illustrates that *H. pylori ureB* is an excellent reporter of in vivo gene expression in *H. pylori*.

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TABLE 1. *H. pylori* urease mutants used in this study

Strain	Analogous strain used for in vitro evaluation[a]	Genotype or description
M6	C57	Wild type
M6Δ*ureB*	412	Replacement of the *ureB* open reading frame with a kanamycin resistance cassette (Δ*ureB*::*kan*)
M6(*cagx-ureB*)	NA[b]	M6{*hpn*::[Φ(P*cagx-ureBcat*)]}, wild-type strain M6 with the recombinant *cag-ureB* fusion integrated 55 bp downstream of the *hpn* stop codon; *x* denotes the specific *cag* promoter region; *cag* promoters used were *cag9* and *cag25*
M6Δ*ureB*(*cagx-ureB*)	585(*cagx-ureB*)	M6Δ*ureB*{*hpn*::[Φ(P*cagx-ureBcat*)]}, M6Δ*ureB* with the recombinant *cag-ureB* fusion integrated 55 bp downstream of the *hpn* stop codon; *x* denotes the specific *cag* promoter region; *cag* genes used were *cag-1*, *-9*, *-13*, *-14*, *-15*, *-21*, and *-25*
M6Δ*ureB*(*hpn-ureB*)	472	M6Δ*ureB*{hpn::[Φ(*hpn-ureBcat*)]}, M6Δ*ureB* with the recombinant *cag-ureB* fusion integrated within the coding region of *hpn*

[a] Detailed descriptions of the mutant constructs and their characteristics in laboratory-passaged *H. pylori* strain C57 have been published (20).
[b] NA, not applicable.

MATERIALS AND METHODS

Bacterial strains and culture conditions. For mouse inoculation experiments *H. pylori* strain M6 (kindly provided by Steven Czinn) was used. This strain was originally isolated from a human patient, colonizes mice, and is easily transformed in vitro. Table 1 summarizes the constructs and terminology used in this report. Construction of the mutants has been previously described in detail (20). M6Δ*ureB*, a deletion mutant of M6 that fails to express urease activity, contains a kanamycin resistance cassette (*aph3'III*) that replaces all but the 35 N-terminal nucleotides of the *ureB* coding sequence (20). A series of seven M6Δ*ureB* derivatives was constructed to contain *cag-ureB* fusions in which *ureB* expression was under control of putative promoters derived from the *cag* pathogenicity island. Fusions were integrated within a putative noncoding region 55 bp downstream of *hpn*, a nickel-binding protein (15). A transcriptional terminator sequence was integrated just upstream of the *cag* promoter sequence to block *hpn*-mediated *ureB* expression. M6Δ*ureB*(*hpn-ureB*) is a derivative of M6Δ*ureB* in which *ureB* was integrated within *hpn*, inactivating that gene and placing the recombinant *ureB* under control of the *hpn* promoter. M6(*cag9-ureB*) and M6(*cag25-ureB*) are M6 derivatives in which the native *ureB* was intact but *cag-ureB* fusions were integrated downstream of *hpn* as described above. The last two strains were included as colonization controls. They express normal urease activity but colonize at a density comparable to that of the urease mutants (about 1 to 4% of M6; see below). Therefore, in vivo urease activity in these strains could be compared directly with in vivo urease activity of the mutants without regard to differences in bacterial colonization density.

Mutant constructs were transferred to mouse virulent M6 by natural transformation. For this, chromosomal or plasmid DNA was isolated by lysis and differential solubilization with a commercially available kit (Qiagen). The recipient strain, *H. pylori* M6 or M6Δ*ureB*, was grown overnight at 37°C in Brucella broth with 10% fetal calf serum in a microaerobic environment with gentle agitation. When bacteria reached mid-log-phase growth (approximately 10^8 to 10^9 CFU/ml), they were diluted 1:100 into 10 ml of fresh Brucella broth. Broths were incubated for 0 to 6 h, approximately 20 to 50 ng of donor DNA was added, and incubation was continued overnight. Each 10-ml broth was then diluted 1:4 in Brucella broth containing selective antibiotics (kanamycin, chloramphenicol, or both) (20 μg/ml) as appropriate. After another overnight incubation, 1-ml aliquots were plated on 5% sheep blood agar plates containing 20 μg of antibiotics per ml as described above. After 4 to 5 days 1 to 500 colonies were visible. Colonies were pooled, plated on selective agar plates as described above, and stored at −70°C in Brucella broth with 15% glycerol until mouse inoculation. Proper insertion of the constructs was confirmed by PCR and verified by urease test before and after animal challenge. Primers used to verify correct insertion of constructs are shown in Table 2. PCR was done by routine methods, and correct insertion was inferred by amplification of a band of the expected size. All of the *cag-ureB* recombinant strains used in animals were derived from the same M6Δ*ureB* isolate by transformation with the relevant DNA constructs. To ensure consistency of colonization data, all transformations were performed at least twice and pools from each transformation were used in animal inoculation.

Urease testing. For quantitation of urease in vitro, the phenol-hypochlorite method was used (25, 37). Briefly, bacteria grown on plates or in Brucella broth were resuspended in 50 mM HEPES, pH 7.5, and diluted (1:20 to 1:1,000, depending on urease activity) into 50 mM HEPES containing 25 mM urea. The dilutions were chosen to ensure that urease activity would be linear for at least 20 min. Suspensions were incubated at 37°C for 20 min, and phenol-nitroprusside solution and hypochlorite solution were added in sequence. The reactions were incubated for 30 min at 37°C and A_{625} was determined. Ammonia was quantified using a standard curve. Urease activity was expressed as nanomoles of ammonia produced per microgram of bacterial protein per minute. Bacterial protein concentration was determined by the Lowry method using a commercially available kit (Sigma). The urease activity of each mutant was based on the average of five to eight separate assays performed with different bacterial preparations on different days.

For quantitation of urease activity in vivo, a modification of the more sensitive ammonia assay kit (Boehringer-Mannheim) was used as previously described (5). Ammonia determination is based on the following linked reactions:

$$\text{Urea} \xrightarrow{\text{Urease}} \text{Ammonium} + CO_2$$

$$\text{Ammonium} + \text{2-Oxoglutarate} \xrightarrow[NADH]{\text{Glutamate dehydrogenase}} \text{L-glutamate} + NAD^+$$

The amount of ammonia produced by urease is proportional to the amount of NAD^+ produced. The assay was modified such that glutamate dehydrogenase was in excess and urease activity was the rate-limiting step (5). Thus, the urease activity was proportional to the rate of NAD^+ production and was measured by a change in optical density at 340 nm. To evaluate urease activity in stomach tissue, gastric mucosal homogenate was washed twice in 50 mM PIPES (piperazine-*N*,*N'*-biz(2-ethanesulfonic acid) buffer, pH 6.8, containing a proteinase inhibitor cocktail (Roche) (1 tablet per ml). The homogenate was resuspended in PIPES and urea was added to a final concentration of 200 μM. Samples were incubated at room temperature for 120 min, and the homogenate was removed by sedimentation in a microcentrifuge. Ammonia concentration in the supernatant was measured by enzymatic assay as described above. Controls contained homogenate with buffer alone (no urea), urea with buffer alone (no homogenate), and uninfected homogenate with buffer (no bacteria). Tissue urease was measured in mice killed 2 weeks after inoculation.

Animals. Female 4- to 6-week-old *Helicobacter*-free C57BL/6 and C57BL/6-*Prkdc*^{scid} (severe combined immunodeficient [SCID]) mice were purchased from Jackson laboratory and were maintained in microisolator cages and fed sterile lab chow and water ad libitum. Germfree $129^{RAGI-/-}$ (recombinase activating gene I knockout [RAG-KO]) mice came from our own colony. C57BL/6 mice

TABLE 2. Primers used to verify *cag-ureB* fusions

Gene	Direction	Sequence
ureB	Reverse	TCT AGA GCC TGT AGT AGG ACC ATA CA
cag-15	Forward	ACA CCC ATT TGA AGC AAA GC
cag-13	Forward	GAT ATG GCT TGT TTG GTG GC
cag-21	Forward	ATC AGG CCT TGA GGC AAA TG
cag-14	Forward	TCG TTG GTT TGT GCT ATA CC
cag-1	Forward	TTC ATA GCC AAA TTC TGC GG
cag-25	Forward	ATT GGT TGT TAC CAC TAG CC
cag-9	Forward	CTT TGC TCA ACA CCT TAT CC
hpn	Forward	CGA GGA TCC GTT GGT TTT AAT CAA GCG

TABLE 3. Colonization by M6 strains and mutants

Bacterial strain	No. of mice colonized/total in each inoculation group			
	C57BL/6 mice killed 2 wks after inoculation	Germfree RAG-KO mice	C57BL/6 mice killed 8 wks after inoculation	Reconstituted SCID mice
M6	10/10	5/5	11/1	2/5
M6Δ*ureB*	0/3	0/4	0/5	ND[a]
M6 transformants				
M6(*cag9-ureB*)	5/5	4/4	ND	ND
M6(*cag25-ureB*)	5/5	ND	ND	ND
M6Δ*ureB* transformants				
M6Δ*ureB*(*hpn-ureB*)	ND	6/6	ND	ND
M6Δ*ureB*(*cag1-ureB*)	11/13	5/5	5/5	2/5
M6Δ*ureB*(*cag9-ureB*)	0/8	0/5	ND	ND
M6Δ*ureB*(*cag13-ureB*)	0/8	ND	ND	ND
M6Δ*ureB*(*cag14*(*ureB*)	0/8	ND	ND	ND
M6Δ*ureB*(*cag15-ureB*)	11/12	5/5	4/4	ND
M6Δ*ureB*(*cag21-ureB*)	0/12	ND	ND	ND
M6Δ*ureB*(*cag25-ureB*)	8/8	5/5	ND	ND

[a] ND, not done.

and germfree mice were orally inoculated with 10^7 CFU of broth-cultured *H. pylori* and killed 2 or 8 weeks after inoculation. SCID mice were inoculated as described above, reconstituted with congenic splenocytes 2 weeks after inoculation, and killed 6 weeks after transfer. RAG-KO mice support high-density colonization by *H. pylori* and were used to determine the in vivo urease level (10). Reconstituted SCID mice develop rapidly progressive severe gastritis in response to infection with *H. pylori* (9, 10), and this model was used to determine the role of urease and bacterial density in eliciting these lesions. The number of animals in each group is given in Table 3.

For animal challenge, bacteria were grown in Brucella broth with 10% fetal calf serum with selective antibiotics as appropriate. Animals were inoculated by oral gavage with 10^7 CFU of mid-log-phase bacteria (about 10^8 to 10^9 CFU of rapidly motile organisms per ml). At sacrifice, stomachs were removed and divided in half longitudinally. One half of each stomach was homogenized in Brucella broth, colonization was determined by plate dilution, and the remainder of the homogenate was used for tissue urease determination. The other half of each stomach from C57BL/6 mice killed 8 weeks after inoculation and from recipient SCID mice was immersed in 10% neutral buffered formalin and embedded in paraffin for histologic examination. Six-micrometer hematoxylin-and-eosin-stained sections were examined and gastritis, neutrophilic infiltration, polymorphonuclear leukocytes (PMN), and metaplasia were quantified as previously described (10). Because gastritis takes at least 8 weeks to develop in *H. pylori*-infected C57BL/6 mice, mice killed 2 weeks after inoculation were not examined histologically.

Statistics. Means were compared by Student's *t* test or Fisher's protected least significant difference to correct for multiple comparisons. Error bars in graphs indicate standard errors of the means.

RESULTS

Urease activity of bacterial mutants in vitro. Urease activity of the bacterial strains in vitro is shown in Fig. 1. The activities of strains with wild-type urease activity [M6, M6(*cag9-ureB*), and M6(*cag25-ureB*)] were not significantly different from each other or from M6Δ*ureB*(*hpn-ureB*). Comparison by pairwise *t* test indicated that the urease activity of all the strains was significantly greater than that of the urease-negative strain M6Δ*ureB*. However, correction for multiple comparisons (Fisher's protected least significant difference) revealed significant differences only between M6Δ*ureB* and the three mutants with the highest urease activity, M6Δ*ureB*(*cag1-ureB*), M6Δ*ureB*(*cag25-ureB*), and M6Δ*ureB*(*hpn-ureB*), in addition to the control strains expressing wild-type urease. Thus, the strains could be classified based on in vitro urease activity into three groups as follows: (i) strains with wild-type urease activity, (ii) mutants with recombinant urease activity that was significantly greater than zero, and (iii) strains with detectable urease activity but for which a statistically significant difference from zero could not be demonstrated.

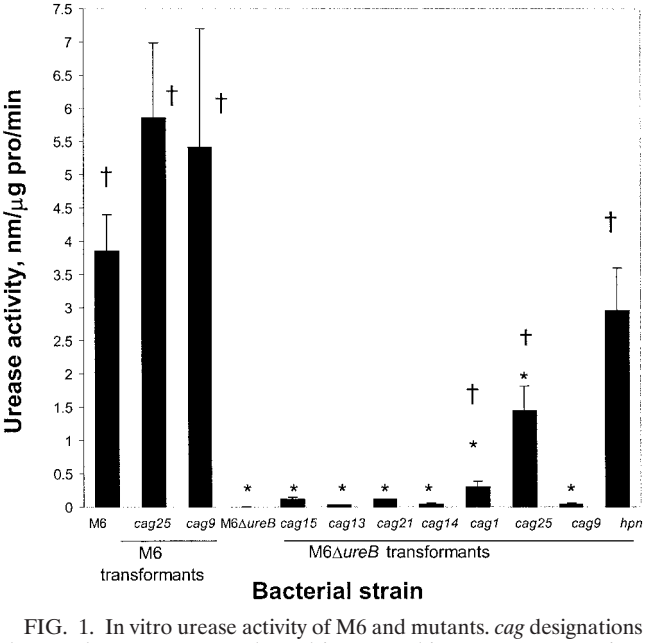

FIG. 1. In vitro urease activity of M6 and mutants. *cag* designations denote the promoter used to drive recombinant *ureB* expression. Strains with wild-type urease activity [M6, M6(*cag9-ureB*), and M6(*cag25-ureB*)] did not differ significantly from each other or from M6Δ*ureB*(*hpn-ureB*). The urease activity of strains M6Δ*ureB*(*cag1-ureB*) and M6Δ*ureB*(*cag25-ureB*) was significantly less than that of the wild type and was significantly greater than zero. Urease activity of the other strains did not differ significantly from that of urease-negative M6Δ*ureB* (see text). ∗, significantly different from M6, $P < 0.05$. †, significantly different from M6Δ*ureB*, $P < 0.05$.

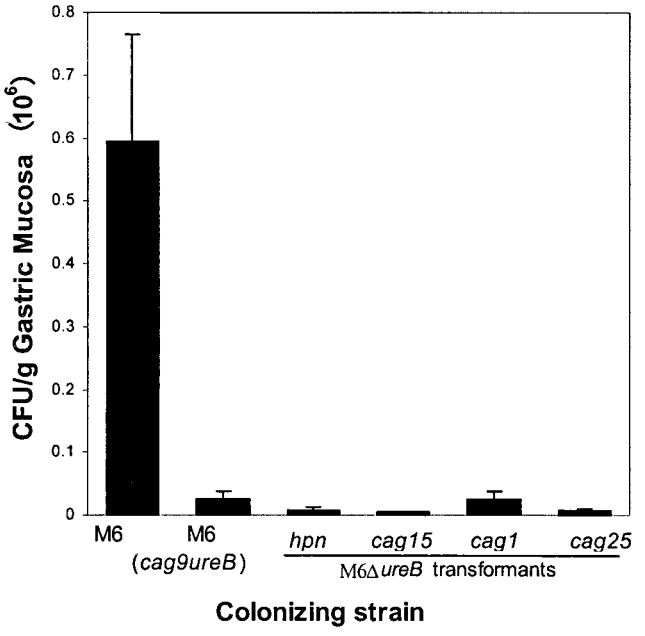

FIG. 2. Colonization density by M6 and colonizing mutants. The colonization densities of all mutants differed significantly from M6 but not from each other ($P < 0.05$).

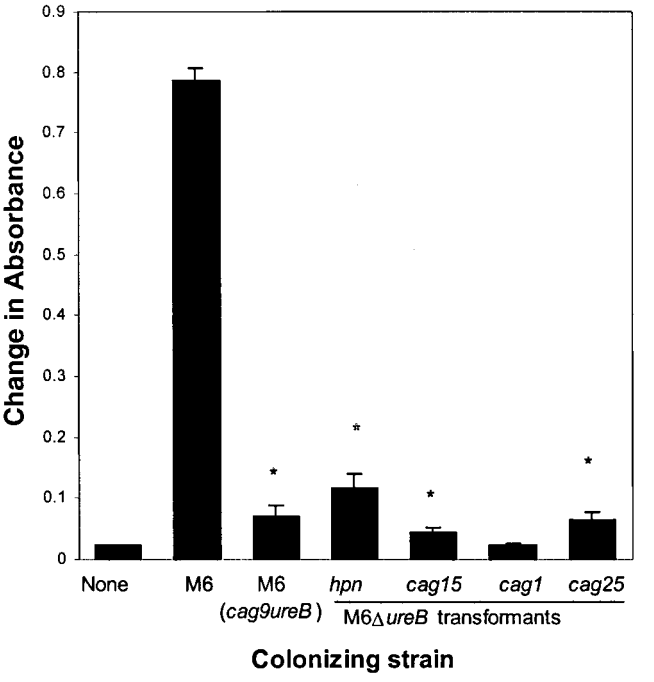

FIG. 3. Urease activity in gastric homogenates from mice colonized by wild-type *H. pylori* strain M6 and colonizing mutants, compared to baseline. Results are expressed as the change in A_{340} over 2 h (see text). All mutant strains are significantly different from M6. Activities of strains M6(*cag9-ureB*), M6Δ*ureB*(*cag15-ureB*), M6Δ*ureB*(*cag25-ureB*), and M6Δ*ureB*(*hpn-ureB*) are significantly greater than those of uninfected gastric homogenate (none) ($P < 0.05$).

Bacterial colonization by wild-type and urease-deficient *H. pylori*. Bacterial colonization of mice by *H. pylori* strain M6 and its mutants and recombinants is shown in Fig. 2. Wild-type strain M6 colonized all mice at a density of 10^6 to 10^7 CFU/g of gastric mucosa, and urease-negative M6Δ*ureB* failed to colonize (not shown). Colonization by mutant strains varied. Of the 11 mutants tested, only the M6 derivatives M6(*cag9-ureB*) and M6(*cag25-ureB*), which expressed wild-type urease, and 4 of the 8 M6Δ*ureB* derivatives colonized mice, as indicated in Fig. 2. All mutant strains, regardless of urease activity or genotype, colonized at a similar density, between 1 and 4% of M6.

Of the four colonizing M6Δ*ureB* transformants, three expressed in vitro urease activity that was significantly different from zero while none of the noncolonizing strains did. The association between high urease production and colonization was significant at a *P* value of 0.0476 (Fisher's exact test). The fourth colonizing strain, M6Δ*ureB*(*cag15-ureB)*, had in vitro urease activity that clustered with the noncolonizing strains. These strains had urease activity which was detectable but was too low to be significantly different from zero using our methods. Thus, strain M6Δ*ureB*(*cag15-ureB)* was in a category by itself. It expressed minimal urease activity in vitro, yet unlike the other low-urease strains, it colonized mice.

In vivo urease activity of wild-type and urease-deficient *H. pylori*. In contrast to differing in vitro urease activity, in vivo urease did not differ between the colonizing strains (Fig. 3). The level of ammonia production by tissue homogenates revealed that urease activity in homogenates colonized by M6 was higher than in homogenates colonized by the mutant strains, as would be expected based on the higher bacterial colonization density. Lower colonization density in the urease-positive colonization control strains was associated with lower in vivo urease activity due to the smaller number of bacteria present. When homogenates with similar bacterial colonization density were compared, however, there were no detectable differences in urease activity between strains. Surprisingly, in vivo urease activity of M6Δ*ureB*(*cag15-ureB*), the mutant with low to undetectable in vitro urease activity, could not be distinguished from that of the other colonizing mutants. In fact, when corrected for colonization density, the in vivo urease activity of M6Δ*ureB*(*cag15-ureB*) was one of the higher activities of the four colonizing mutants (Fig. 4).

Gastritis due to wild-type and urease-deficient *H. pylori*. Gastritis in mice infected with *H. pylori* strain M6 and colonizing mutants was typical of *H. pylori* gastritis in mice. In C57BL/6 mice killed 8 weeks after inoculation, lesions were moderate in extent, most commonly involving less than 20% of the gastric mucosa (Fig. 5). They were characterized by lymphocytic, plasmacytic, and neutrophilic infiltrates which were multifocal in distribution and mild to moderate in severity (Fig. 6). Foci of severe inflammatory infiltrates were accompanied by gastric epithelial metaplasia and were characterized by loss of normal fundic gland morphology and replacement by undifferentiated glands lined by mucus-type epithelium. In C57BL/6 mice, gastric lesions were most extensive in mice colonized by strain M6 but were also present in mice colonized by the two urease transformants. Differences in the extent of lesions between groups did not reach statistical significance (Fig. 5).

In recipient SCID mice killed 6 weeks after transfer, gastritis was more extensive than in C57BL/6 mice. Up to 100% of the

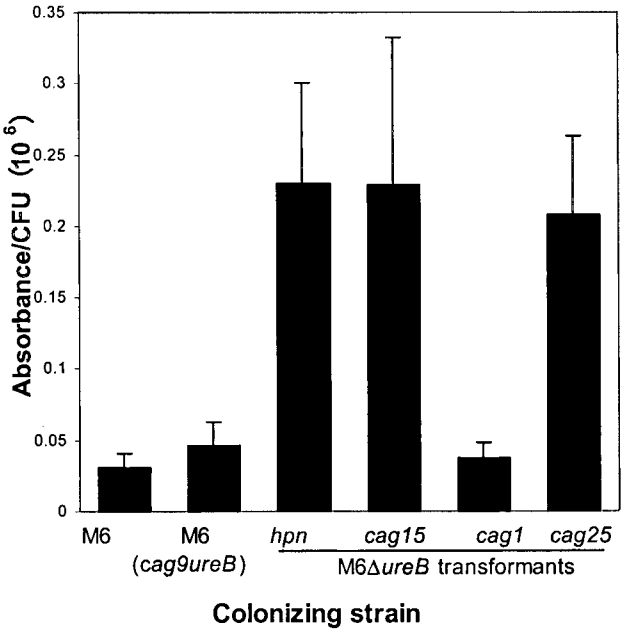

FIG. 4. Urease activity in gastric homogenates corrected for colonization density. Correcting for the number of bacteria per gram of gastric mucosa did not reveal any significant differences in the in vitro urease activity of the different bacterial mutants.

gastric mucosa was affected and metaplasia was common (Fig. 7). Like C57BL/6 mice, there was no difference in the extent of gastritis or neutrophilic infiltrate between mice infected with M6 and those infected with M6Δ*ureB(cag1-ureB)*, in spite of a difference of up to 100-fold in bacterial density and 35-fold in in vivo urease activity (see Fig. 2 and 3). Histologic lesions in

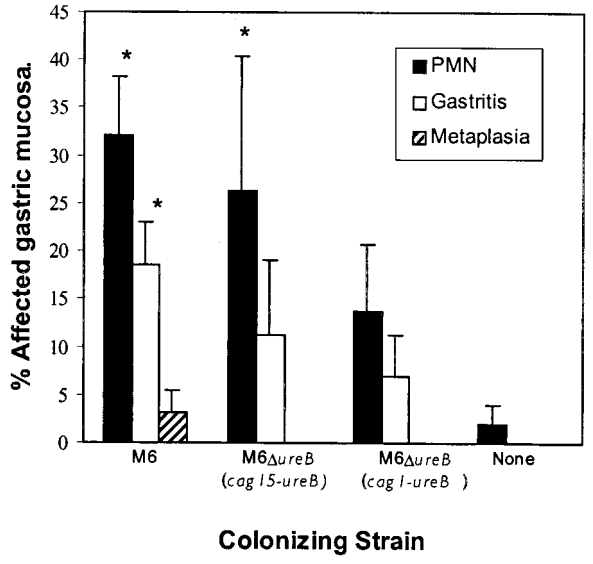

FIG. 5. Extent of gastric lesions in C57BL/6 mice killed 8 weeks after infection (scored as described in Materials and Methods). None, uninfected mice; *, significantly different from uninfected mice, $P < 0.05$. There were no significant differences between the three infected groups.

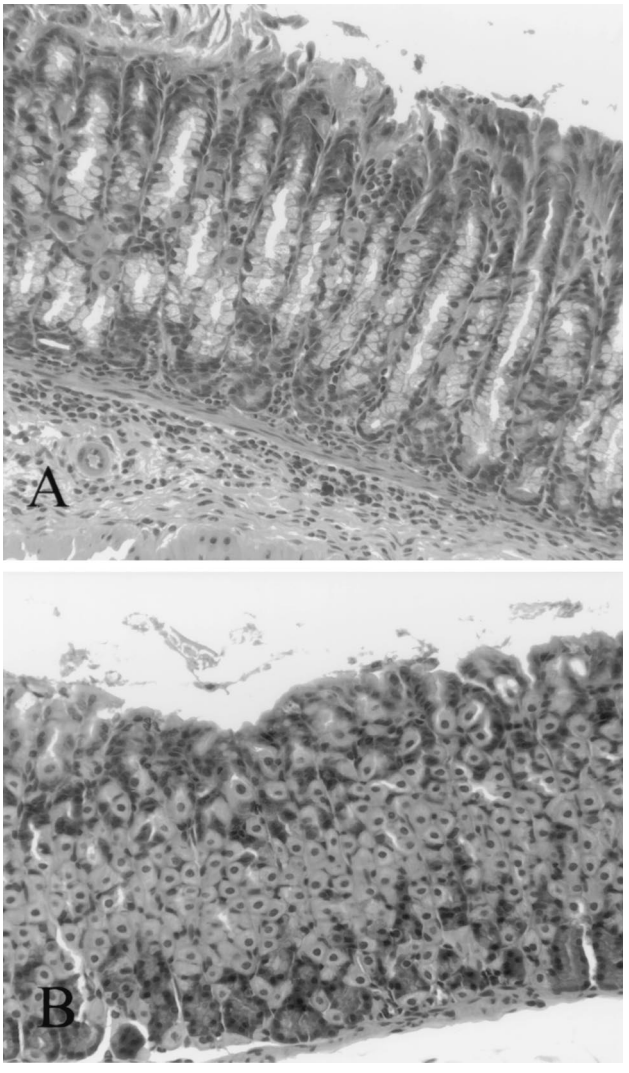

FIG. 6. (A) Fundic gastric mucosa from a C57BL/6 mouse infected with *H. pylori* strain M6Δ*ureB(cag15-ureB)* and killed 8 weeks after inoculation. Mild gastritis characterized by mixed lymphocytes and neutrophils with a few plasma cells is present at the base of the gastric glands. (B) Uninfected control mouse. No inflammatory cells are present.

recipient SCID mice were similar to those in C57BL/6 mice but were more severe (Fig. 8).

DISCUSSION

The results of this study demonstrate that restoration of urease activity to urease-deficient *H. pylori* can restore colonization ability. This constitutes the first definitive proof of the essential role of urease in colonization by *H. pylori*. In addition, these results suggest that there is a minimum threshold of urease activity necessary for colonization. Of the four recombinants that colonized mice, three had the highest in vitro urease activity, which was significantly greater than that of the urease-negative mutant, although it was less than that of the wild type. The five noncolonizing recombinants all had low in vitro urease activity. In these strains urease activity was detect-

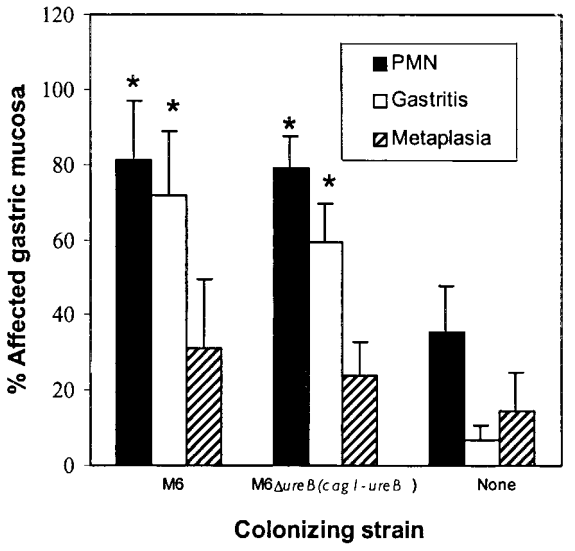

FIG. 7. Gastric lesions in recipient SCID mice were more extensive than in C57BL/6 mice, but like C57BL/6 mice there were no significant differences in the extent of lesions in the two infected groups. None, uninfected mice; *, significantly different from uninfected mice, $P < 0.05$.

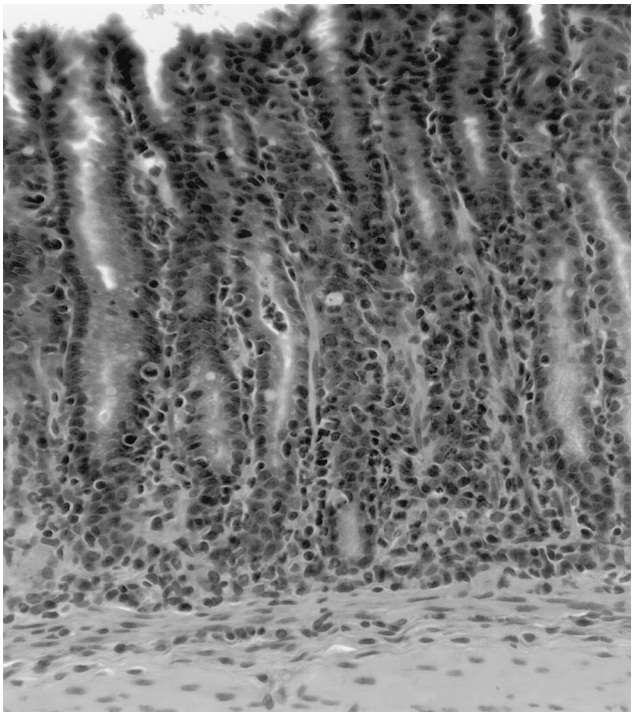

FIG. 8. Fundic gastric mucosa from a recipient SCID mouse infected with *H. pylori* strain M6 and killed 6 weeks after transfer. Marked gastritis characterized by mixed lymphocytes, neutrophils, plasma cells, and histiocytes is accompanied by loss of the normal fundic glands and replacement by less-differentiated mucus-type glands.

able, but our assays were insufficiently sensitive to distinguish these strains statistically from the urease-negative mutant.

A surprising exception to this observation was strain M6Δ*ureB(cag15-ureB)*. In vitro, the urease activity of this mutant clustered with that of the noncolonizing mutants. However, the strain was able to colonize and urease assays of gastric homogenates infected with M6Δ*ureB(cag15-ureB)* were indistinguishable from those of homogenates infected with the other colonizing strains. In fact, all five colonizing mutants tested, M6(*cag9-ureB*), M6Δ*ureB*(*hpn-ureB*), M6Δ*ureB*(*cag1-ureB*), M6Δ*ureB*(*cag15-ureB*), and M6Δ*ureB*(*cag25-ureB*), had similar in vivo urease activities. Taken together, these findings strongly suggest that urease activity in M6Δ*ureB(cag15-ureB)* is upregulated in vivo, thus allowing sufficient urease expression to support colonization. These results are further supported by previously reported in vitro findings indicating that *cag-15* is upregulated in response to contact with epithelial cells in vitro (20). The function of Cag-15 remains unknown and the protein lacks known homology. However, both in vitro and in vivo evidence of upregulation suggests that it may represent an important *H. pylori* colonization or virulence factor.

Interestingly, colonization density by all the mutants, including the positive control strains [M6(*cag9-ureB*) and M6(*cag25-ureB*)], was lower than colonization density by M6. We previously determined that insertional mutagenesis of *hpn* itself decreases colonization density by *H. pylori* (not shown), accounting for the decreased colonization by strain M6Δ*ureB*(*hpn-cag*), but the other mutants expressed *hpn* and the diminished colonization could not be attributed to effects on *hpn* itself. The loss of colonization ability with insertion of the *cag-ureB* fusions is most likely due to the location of insertion (downstream of *hpn*). According to the published *H. pylori* gene sequences (35), the insertion did not interrupt any known open reading frame (20), but it is possible that M6 differs from published strains or that this chromosomal region may contain a regulatory or other sequence important to *H. pylori* survival in vivo. Diminished colonization density could not be attributed to loss of the wild-type *ureB* because even strains with wild-type urease activity expressed lower colonization in the presence of the *cag-ureB* insertion. In addition, in vivo manipulation is unlikely to account for differences in colonization by the mutants. We and others have shown that some *H. pylori* mutants colonize with an efficiency equal to that of the wild type in spite of in vitro manipulations which were similar to the ones used in this study (6, 7, 31). In addition, we used pools of mutants and repeated the transformations at least twice to ensure that random genetic events in a single clone would not result in spurious loss of colonization potential. Whatever the pathogenesis, the decrease in colonization density was consistent and similar for all strains which contained fusion constructs and thus was likely unrelated to urease activity.

In addition to their implications for the role of urease and *cag-15* in colonization, the results reported here also have implications for the study of *H. pylori* in areas other than urease pathogenesis. First, this is the first demonstration of in vivo complementation of any *H. pylori* virulence factor. We have demonstrated that in vivo complementation can be successful in restoring enzyme activity and is a good method to demonstrate unequivocal colonization dependence of a specific bacterial factor. Previous studies showing that urease-negative mutants fail to colonize (2, 5, 8, 36) provided strong evidence that urease was needed for growth in vivo. However,

definitive proof awaited the demonstration that loss of colonization could be restored specifically by replacement of urease activity. Success in complementation of urease activity in vivo indicates that this method can be useful in evaluating the role of other putative colonization factors for *H. pylori*. In addition, we have shown that urease is an excellent in vivo reporter gene. Expression of *ureB* under control of *H. pylori cag* promoters indicates not only that recombinant *ureB* can restore colonization ability but also that some *cag* genes are expressed in vivo at a level sufficient to allow colonization. Further, in combination with the previously published in vitro observations (20), the results are highly suggestive that at least one gene, *cag-15*, is upregulated in vivo.

We did not demonstrate differences in gastritis associated with urease activity in this study. In C57BL/6 mice this was not surprising given the relatively mild gastritis characteristic of *H. pylori* infection of these mice 8 weeks after inoculation (10). However, the similarity in lesions in recipient SCID mice colonized by the different strains was striking. We have shown that recipient SCID mice rapidly develop severe chronic active gastritis with epithelial metaplasia in response to infection by *H. pylori* (10). In these animals up to 100% of the gastric mucosa is inflamed 4 to 6 weeks after transfer, and in some mice normal gastric fundic mucosa is virtually absent. This model represents a robust method of evaluating the host response to *H. pylori* antigens and therefore was used in this study. Even in this strong host response model, however, there was no difference between mice infected with wild-type and urease-deficient *H. pylori*, in spite of differences in in vitro urease activity, absolute urease activity in vivo, and colonization density. This is strong evidence that neither urease activity nor bacterial colonization density are primary inducers of gastritis and gastric epithelial damage due to *H. pylori*.

Because of the similarity of urease activity in vivo in all the colonizing mutants we could not definitively distinguish the effects of urease activity per bacterium from those of decreased activity per gram of gastric mucosa. However, clearly total urease activity does not influence severity of gastritis in this model whether the difference is due to inherent urease activity of the colonizing strain or bacterial burden alone. We have previously shown that there is no direct toxic effect of large numbers of colonizing urease-positive bacteria in the absence of a host response (10). Taken together, these studies strongly suggest that while urease is an essential colonization factor for *H. pylori* it does not contribute to severity of disease.

In summary, we have confirmed through complementation that urease is essential for colonization and that urease is an excellent reporter gene for in vivo expression of *H. pylori* promoters. In addition, our results suggest that at least one of these promoters, *cag-15*, is upregulated in vivo. Finally, we have demonstrated that, at least in mice, neither total urease activity nor bacterial colonization density contributes to gastritis due to *H. pylori*.

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