INFECTION AND IMMUNITY, July 1991, p. 2470–2475
0019-9567/91/072470-06$02.00/0
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Vol. 59, No. 7

Essential Role of Urease in Pathogenesis of Gastritis Induced by *Helicobacter pylori* in Gnotobiotic Piglets

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Received 4 March 1991/Accepted 2 May 1991

A mutant strain of *Helicobacter pylori* with weak urease activity was created by using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. The urease activity of the mutant (0.036 ± 0.009 nmol of urea per μg of bacterial protein per min) was 0.4% of that of the parental strain (8.20 ± 2.30 nmol of urea per μg of bacterial protein per min). The mutant was otherwise indistinguishable from the parental strain. Both demonstrated prominent catalase and oxidase activities, and both produced vacuolating cytotoxin. Restriction endonuclease and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) patterns and ultrastructure were identical for the two strains. The mutant was fully motile, as evaluated by spreading in soft agar and by direct microscopic examination. Growth rate and colony size and morphology were identical for the mutant and parental strains. Seventeen gnotobiotic piglets were challenged with either the mutant or the parental strain and sacrificed 3 or 21 days after challenge. Gastric tissue was examined histologically and cultured for *H. pylori*. Of seven piglets challenged with the parental strain, all became infected. *H. pylori* was not recovered from any of 10 piglets challenged with the urease-negative strain. Lymphofollicular gastritis was present in all seven piglets challenged with the parental strain but in none of the piglets challenged with the urease-negative strain. These results suggest that prominent urease activity is essential for colonization by *H. pylori*.

A variety of virulence factors have been proposed for *Helicobacter pylori*. Among these are motility (3), exotoxin (12), mucinase (18), adhesion (19), and urease activity (7). Although convincing arguments have suggested mechanisms by which these factors may contribute to virulence, there is little direct evidence linking them to virulence in humans or gnotobiotic piglets. We demonstrated that motility is a virulence factor for *H. pylori* (3), but other virulence factors have not been explored.

The marked urease activity of *H. pylori* suggests that this enzyme contributes to the ability of the organism to colonize the acidic environment of the stomach by providing an alkaline microenvironment (7). If this is correct, urease would be essential for colonization. The goal of this study was to evaluate the role of urease in the ability of *H. pylori* to colonize the stomachs of gnotobiotic piglets. In this paper, we describe a mutant strain of *H. pylori*, created with nitrosoguanidine, which has minimal urease activity. We compare the activity at saturating urease concentration (maximum urease activity) and apparent affinity constant (K_a) of urease from this strain with those from strains of known virulence for gnotobiotic piglets. In addition we evaluate the virulence of this mutant strain in gnotobiotic piglets.

MATERIALS AND METHODS

Bacterial strains. Six strains of *H. pylori* were used. Strains 26695, 60190, and Tx30a are laboratory-adapted strains used in previous virulence studies in gnotobiotic piglets (3). Strains 89-1641 and 87-3887 were recovered from gnotobiotic piglets challenged with strain 26695. Strain 26u1 was produced by mutagenesis of strain 87-3887. All strains were evaluated for production of catalase, oxidase, and cytotoxin as previously described (3).

Mutagenesis. Strain 87-3887 was used as the parental strain. Bacteria were grown in broth to mid-logarithmic phase (approximately 5×10^8 CFU/ml), sedimented at 3,000 × *g* for 10 min, and resuspended in sterile phosphate-buffered saline to a concentration of approximately 5×10^8 cells per ml. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (Sigma Chemical Co., St. Louis, Mo.) was added to a final concentration of 25 μg/ml, and bacterial suspensions were incubated for 10 min in 10% CO_2. Aliquots were immediately diluted into 10 volumes of brucella broth and incubated at 37°C in 10% CO_2 for 8 h. The aliquots were then plated in 0.5% brucella agar (soft agar) to give a final concentration of 100 to 200 colonies per plate and were incubated for 4 to 5 days.

Identification of urease-negative mutants. Urease broth concentrate (BBL, Cockeysville, Md.) was diluted 1:5 in sterile 1.5% methylcellulose (Dow Chemical Corp.) and then layered onto petri dishes containing mutagenized *H. pylori* colonies embedded in soft agar. Most of the colonies turned pink within 5 to 10 min, indicating strong urease activity, but occasional colonies remained white. White colonies which also demonstrated spreading in the agar (i.e., which were motile) were harvested with a sterile swab and streaked onto blood agar plates for further analysis. In this way motile colonies which produced little or no urease could be identified and isolated.

Urease assay. For urease analysis, bacteria were grown in brucella broth to stationary phase, harvested by centrifugation in a Sorvall RC2-B refrigerated centrifuge at 3,000 × *g* for 10 min, washed twice in sterile distilled water, counted with a hemacytometer, and resuspended in sterile distilled water to a concentration of 10^9 bacteria per ml. In our laboratory, enumeration of *H. pylori* with a hemacytometer has proven to be a reliable estimate of CFU (data not shown). Bacterial suspensions were used fresh or frozen at −20°C for up to 2 weeks. Urease activity was measured by an adaptation of the method of Ferraro (5, 8). Reagents used were as follows. NADH and triethanolamine buffer, pH 8.0, with 2-oxoglutarate were from a commercially available urea

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and ammonia assay kit (Boehringer-Mannheim, Indianapolis, Ind.). Glutamate dehydrogenase (GDH) was from Sigma or Boehringer-Mannheim. The reaction mix contained 33.0 μg of NADH per ml, 730.0 μg of 2-oxoglutarate per ml, and 49 to 52 U of GDH per ml in a final volume of 200 μl of triethanolamine buffer, pH 8.0. Except in the determination of apparent Michaelis constant (K_a), the reaction mix contained 200 mM urea. All reactants were brought to room temperature. Bacteria (approximately 5×10^7/ml in water) were added, and the reaction was read at 340 nm in a 96-well microtiter plate (Dynatech) by using a UVMax kinetic microplate reader (Molecular Systems, Menlo Park, Calif.). For measurement of urease from strain 26u1, 10^{10} bacteria per ml were used. For standardization of the assay, purified jackbean urease (Boehringer-Mannheim) was used. Preliminary studies to determine optimal concentration for all reagents were done. Concentrations were chosen such that urease activity was the rate limiting step in the range of urease activity used in the assay (2.0 to 4.0 nmol of urea hydrolyzed per min). For this purpose it was necessary to increase the GDH activity to 49 to 52 U/ml (approximately 10-fold greater than the activity recommended by the kit manufacturer). Under these conditions, rate of NADH oxidation increased in a linear fashion as urease activity increased, and doubling the GDH concentration had no effect on rate of NADH oxidation. There was no oxidation of NADH in the absence of either urea or bacteria. For determination of K_a of bacterial urease, the rate of urease hydrolysis was measured at five different urea concentrations of between 1.0 and 8.0 mM. Urea concentration was limiting at all five concentrations. Urease activity at saturating urea concentration (maximum urease activity) and K_a were calculated by using the Eadie-Hofstee transformation (11). Calculated in this way, maximum urease activity for all strains corresponded to those measured by using the same concentration of bacteria at a high substrate concentration (200 mM).

Analytical methods. Total protein in bacterial samples was determined by using Peterson's modification of the method of Lowry (17). Endonuclease restriction with *Hin*dIII was performed as previously described (3). Profiles of total bacterial proteins were generated and analyzed by using methods previously described (16). Briefly, bacterial proteins were separated by electrophoresis on 10% polyacrylamide gels (10). Gels were scanned with an Ultroscan XL laser densitometer (LKB Pharmacia, Piscataway, N.J.). Protein patterns were compared by cluster analysis with customized computer software. The relatedness of bacterial strains is expressed as percent similarity. On the basis of previous studies (16), bacterial strains having greater than 90% similarity are considered identical.

Growth curves. Bacteria were grown in brucella broth as previously described (14). Flasks containing 25 ml of brucella broth were inoculated with approximately 10^6 bacteria from a population in logarithmic growth. Each curve was done in triplicate, and the results were expressed as the mean of three trials. Flasks were sampled at 6-h intervals. Samples were enumerated by hemacytometer count and confirmed by standard plate count.

Ultrastructure. Bacteria were grown in brucella broth, washed, stained with 1% phosphotungstic acid and examined by using a Phillips 300 transmission electron microscope, as previously described (3).

Animal model. Seventeen outbred, Yorkshire cross piglets from six litters were delivered by cesarian section and maintained in sterile isolation units as previously described

TABLE 1. Urease activity of *H. pylori* strains

Strain	Maximum activity[a]	K_a[b] (mM)
Laboratory		
26695	5.03 ± 1.3	1.27 ± 0.10
60190	16.27 ± 5.72	1.02 ± 0.10
Tx30a	7.23 ± 1.2	1.13 ± 0.16
Porcine		
89-1641	14.30 ± 4.61	1.20 ± 0.10
87-3887	8.20 ± 2.30	1.05 ± 0.19
Urease mutant, 26u1	0.036 ± 0.009[c]	1.43 ± 0.19

[a] Expressed as nanomoles of urea hydrolyzed per μg of bacterial protein per minute.
[b] No significant difference between groups.
[c] Significantly different from parental strain, 87-3887 ($P = 0.0063$, Student's *t* test). Significantly different from all other groups ($P = 0.014$, Fischer's least significant difference).

(9). They were divided into experimental groups as follows. Piglets in group A (seven piglets) were challenged with strain 87-3887 (the parental strain). Groups B (six piglets) and C (four piglets) were challenged with 26u1 (the urease-negative mutant). Piglets in groups A and B were sacrificed 21 days after challenge, and piglets in group C were sacrificed 3 days after challenge. At sacrifice, gastric tissue was collected for culture and histopathology as previously described (3, 4). Piglets with gastritis were distinguished by lymphoplasmacytic infiltrates and lymphoid follicles in the gastric mucosa (1, 4, 9). Piglets without gastritis had no inflammatory cell infiltrates in the gastric mucosa. To determine bacterial colonization rate, gastric mucosa from infected piglets was removed, weighed, and homogenized, and 10-fold serial dilutions of the homogenates were inoculated onto blood agar plates for enumeration.

Statistics. All urease test results are reported as mean and standard error of at least six trials. Sample means were compared by Student's *t* test or by one-way analysis of variance. Homogeneity groups were determined by using Fischer's least significant difference. Growth rates were compared by using a general linear model for multiple regression (15).

RESULTS

Mutagenesis. Mutagenesis of strain 87-3887 with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine produced rare colonies (less than 1/1,000) whose urease activity was undetectable by our urease broth overlay method. One of these, strain 26u1, showed consistently weak urease activity. As shown in Table 1, the urease activity of strain 26u1 was 0.4% of that of the parental strain, 87-3887. There was no significant difference in the urease activity of any of the other strains of *H. pylori* tested. Thus, the mutant strain showed urease activity which, although present, was uncharacteristically low compared with that of other strains of *H. pylori*.

Urease quantitation. Maximum urease activity of all strains was expressed as nanomoles of urea hydrolyzed per microgram of bacterial protein per minute. Urease activities of fresh bacterial samples (15.5 ± 14.9 nmol of urea hydrolyzed per μg of protein per min; $n = 4$) did not differ from those of samples frozen for up to 2 weeks (32.8 ± 26.7 nmol/μg/min; $n = 4$).

There was no difference in the urease activity of any of the naturally occurring strains tested (Table 1). Thus, even after

TABLE 2. Virulence of urease-positive and urease-negative *H. pylori* for gnotobiotic piglets

Group and no. of piglets	Bacterial strain	Days after challenge	No. infected[c]	No. with gastritis
A (7)	87-3887[a]	21	7	7
B (6)	26ul[b]	21	0	0
C (4)	26ul	3	0	0

[a] Urease-positive parental strain.
[b] Urease-negative mutant strain.
[c] Number from which *H. pylori* was recovered.

prolonged laboratory passage of strains 26695, 60190, and Tx30a, there was no loss of urease activity compared with that in fresh porcine isolates, strains 87-3887 and 89-1641. Conversely, there was no increase in urease activity in strains recovered from piglets (strains 87-3887 and 89-1641) compared with that in the challenge strain, 26695.

The urease activity of the mutant strain, 26ul, was significantly less than that of all other strains. In this strain urease was undetectable by routine screening methods. When quantitative methods were used, activity was detectable, but minimal (only 0.4% of that of the parent strain) (Table 1). K_a did not differ for any of the strains tested, including the urease mutant (Table 1). However, the high concentration of bacteria necessary for detection and the low rate of urea hydrolysis by the mutant strain hindered exact determination of K_a for this strain. In this study, K_a, measured with bacterial cell suspensions, differed somewhat from published values for the actual Michaelis constant (K_m), measured by using purified urease from *H. pylori* (0.3 mM [2] and 0.8 mM [13]).

Comparison of mutant and parental strains. Other than urease activity, the mutant strain did not differ from the parental strain. Both strains demonstrated prominent catalase and oxidase activities, detectable by routine screening methods. Both produced vacuolating cytotoxin. Colony morphology and growth rate of the two strains were identical (Fig. 1). The slope of the linear regression line for strain 26ul growth (0.105) was not significantly different from that of strain 87-3887 (0.113; $P < 0.05$). The slopes corresponded to doubling times of 2.85 h for strain 26ul and 2.65 h for strain 37-3887. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of total bacterial protein from urease-negative and parental strains revealed similar patterns. Densitometer scans of total bacterial protein profiles from both strains were identical (Fig. 2). Cluster analysis of the densitometer scans revealed greater than 94% similarity between the two strains, suggesting that the strains are identical. Restriction endonuclease patterns were identical for the mutant and parent strains (Fig. 3). The mutant was fully motile, as demonstrated by spreading in soft agar and by direct microscopic examination. In addition, ultrastructural examination of broth cultures of both strains revealed short, curved rods with a polar tuft of sheathed flagella (Fig. 4). Thus, the mutant strain had minimal urease activity but otherwise did not differ from the parental strain.

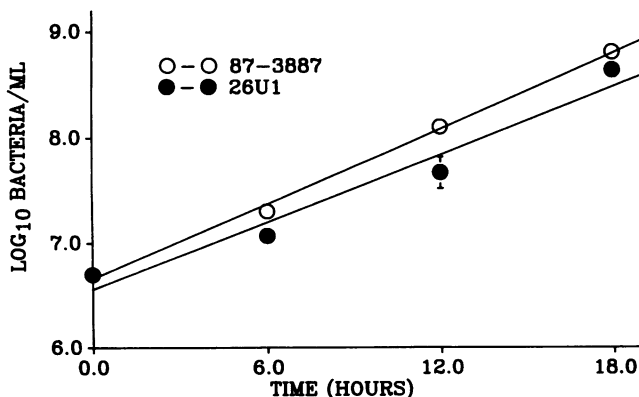

FIG. 1. Growth curves for urease-negative mutant (26ul) and parental (87-3887) strains of *H. pylori*. Lines represent least-squares regression. Vertical bars represent standard deviation. Slopes of the two lines are not significantly different ($P < 0.05$).

Virulence of urease-negative *H. pylori*. To compare the virulence of the two strains, gnotobiotic piglets were challenged with either the parental or the urease-negative strain (Table 2). When piglets were sacrificed 21 days after challenge, *H. pylori* was recovered from all seven piglets challenged with parental strain 87-3887 but from none of six piglets challenged with mutant strain 26ul. In those piglets colonized, the bacterial colonization rate ranged from 8.0×10^4 to 2.0×10^6 CFU/g of gastric mucosa. Recovered bacteria demonstrated prominent urease activity. In addition, histologic gastritis was present in all seven piglets challenged with strain 87-3887 but in none of those challenged with strain 26ul. To determine whether urease-negative *H. pylori* colonized but did not persist, piglets were challenged with urease-negative *H. pylori* and sacrificed 3 days after challenge. Three days was chosen because previous studies have shown that colonization by wild-type *H. pylori* is established by that time (unpublished data). *H. pylori* was not recovered from any of the four piglets challenged with urease-negative *H. pylori* and sacrificed 3 days after challenge.

DISCUSSION

In order to evaluate the role of urease in bacterial colonization, we isolated and characterized a mutant strain of *H. pylori* with minimal urease activity and evaluated the virulence of this strain in gnotobiotic piglets. Except for urease activity, the mutant strain did not differ from the parental strain. Protein profiles, endonuclease restriction patterns, ultrastructure, and in vitro growth characteristics were the same for the two strains. Greater than 94% similarity of total protein content between parental and mutant strains indicated identity between these strains. Catalase and oxidase, two enzymes used for identification of *H. pylori*, were prominent in both the parent and the mutant strains. Both strains produced vacuolating cytotoxin. Thus, although the presence of undetected mutations cannot be ruled out, the two strains are indistinguishable on the basis of the methods used here.

The urease-negative mutant strain was unable to colonize gnotobiotic piglets, in contrast to the parental strain, which was fully virulent. The close correlation between urease activity and virulence, demonstrated here, has not been demonstrated for any other virulence factor of *H. pylori*. A previous study correlated motility with virulence, but the strains used were unrelated and differed in endonuclease restriction pattern (3). Thus, unidentified differences between strains could have accounted for differences in virulence in that study. In this study, the creation of a urease-negative mutant of a virulent strain circumvented that ambiguity. Thus, the inability of strain 26ul to colonize

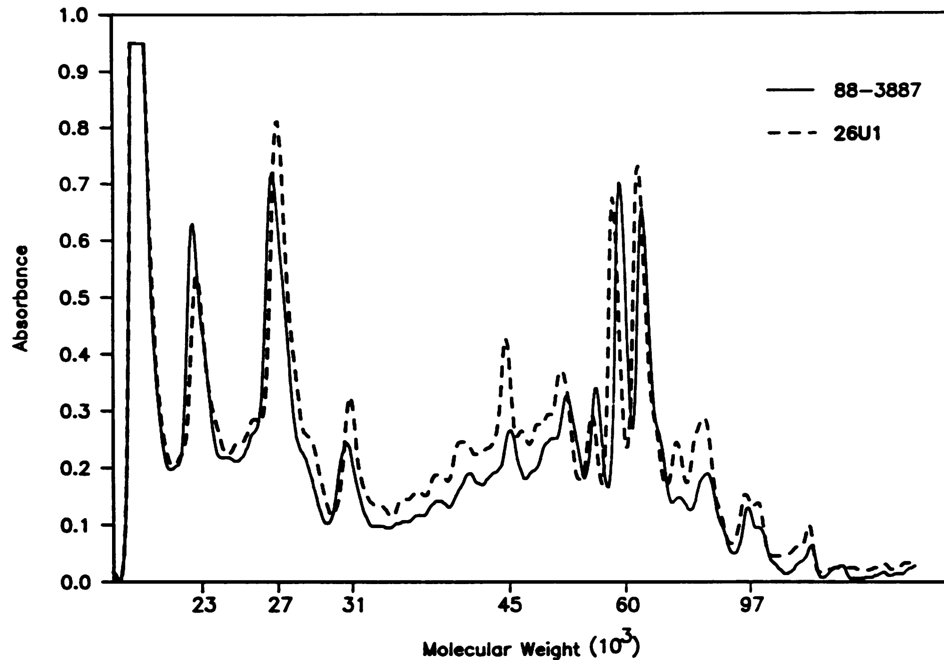

FIG. 2. Densitometry scans of SDS-PAGE of total bacterial protein from urease-negative mutant (26ul) and parental (87-3887) strains of *H. pylori*. All detectable protein species are present in similar concentrations in both strains.

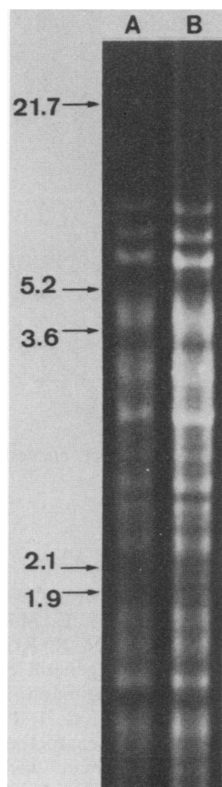

FIG. 3. *Hin*dIII restriction endonuclease digests of total bacterial DNA from urease-negative mutant (A) and parental (B) strains of *H. pylori*. Size markers (in kilobases) are indicated.

gnotobiotic piglets is closely correlated with the loss of urease activity, demonstrating that prominent urease activity is essential for colonization by *H. pylori*.

These data indicate that urease activity is a colonization factor for *H. pylori*. However, the precise role of urease in promoting colonization is not known. In this study, viable bacteria were not detected even as early as 3 days after challenge with a urease-negative strain. This suggests that urease is essential for early colonization, possibly allowing the acid-sensitive bacteria to reach the relatively alkaline colonization site below the gastric mucus (7). Studies directed at determining the early kinetics of colonization by urease-negative and wild-type *H. pylori* and the effect of modulation of host gastric pH will further elucidate the role of urease in colonization.

There are several possible interpretations of our observations. It is possible that urease is incidental to colonization potential of *H. pylori* and that the failure of 26ul to colonize is due to an undetected second mutation. Such mutations may occur particularly with nitrosoguanidine, which may produce multiple mutations in localized regions (1). Such mutations cannot be completely ruled out by our methods. However, the fact that the growth characteristics are the same in the mutant and parental strains suggests that secondary mutations, if present, may not be significant for bacterial growth.

There are other indications that urease is important for gastric colonization. Most of the bacterial species isolated from the stomach have prominent urease activity (6). Furthermore, the persistence of urease activity in the laboratory-adapted strains in this study (strains 26695, 60190, and Tx30a) suggests that such activity is not easily lost in vitro, despite the fact that it is not necessary for growth outside the host. In addition, the similarity in urease activity in laboratory-passaged strains (26695, 60190, and Tx30a) and porcine isolates (89-1641 and 87-3887) suggests that urease activity in

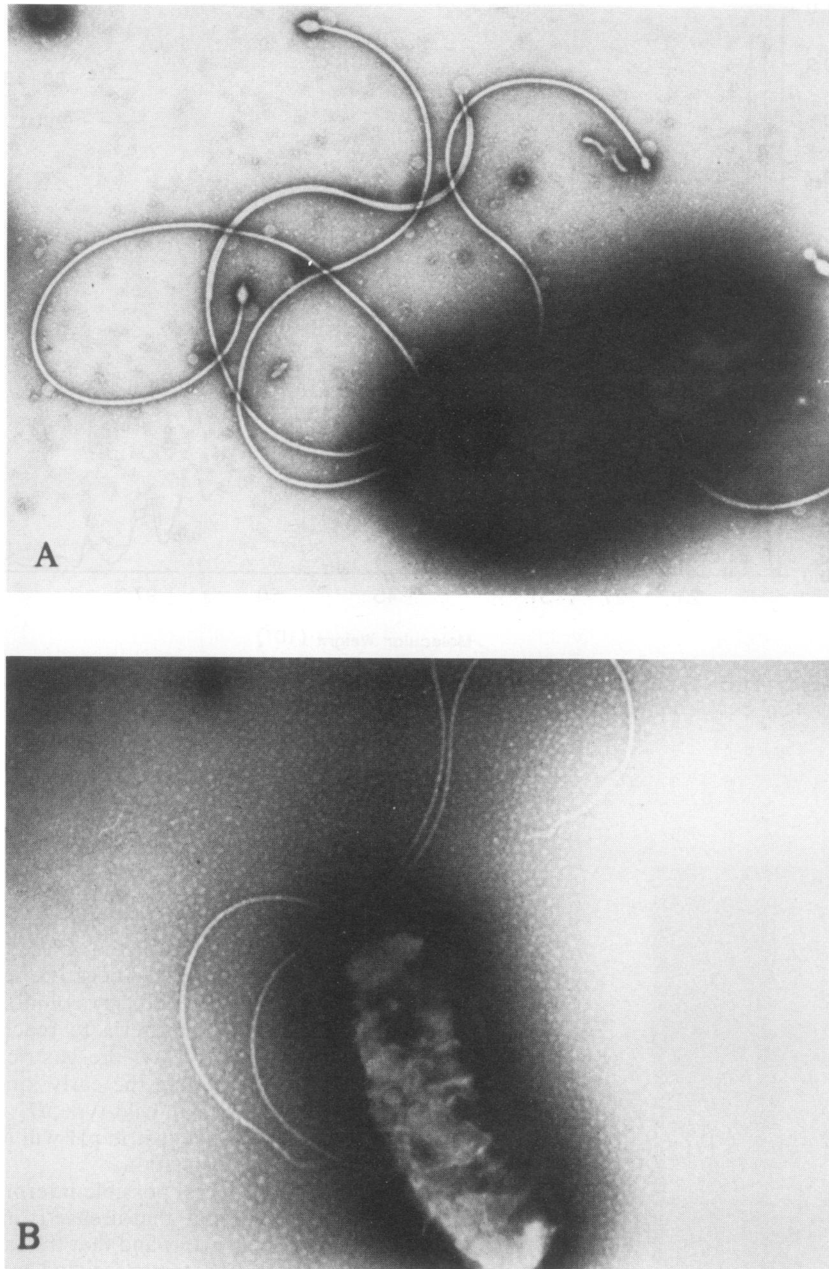

FIG. 4. Ultrastructure of parental (A) and urease-negative mutant (B) strains of *H. pylori*. Both are short, curved rods with a polar tuft of sheathed flagella.

this organism is constitutive and is not regulated by growth conditions. This, in combination with the large proportion of metabolic energy expended on urease by the bacterium, suggests that prominent urease activity is indispensable for survival of *H. pylori* in the gastric microenvironment.

ACKNOWLEDGMENTS

We thank Robert D. Leunk and Emma Williams for help in preparing the densitometry scans and Judy Dubena, Nancy Hughey, and Susan Ringler for technical help.

This work was supported by Public Health Service grant DK39570-01A3 from the NIH. K. A. Eaton was supported by NIH grant AI07938-02.

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