

## *Campylobacter pylori* Virulence Factors in Gnotobiotic Piglets

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**Thirty-three gnotobiotic piglets from four litters were challenged with motile and nonmotile strains of *Campylobacter pylori*. The most motile strain, 26695, was the most virulent, with a 100% infection rate. The least motile strain, Tx30a, was the least virulent, with an infection rate of only 17%. Strain 60190 was weakly motile and had intermediate virulence, with an infection rate of 40%. Strains recovered from piglets were more motile than the challenge strains. The challenge strains also differed in cytotoxin production. The least virulent strain, Tx30a, was nontoxicogenic, while the other two strains produced high levels of cytotoxin. Thus, virulence of *C. pylori* for gnotobiotic piglets correlated very well with motility and not as well with cytotoxin production.**

*Campylobacter pylori* is a newly described bacterial pathogen which has been associated with gastritis and peptic ulcers in humans. There is a strong clinical correlation between histologic evidence of gastritis and isolation of *C. pylori* from the gastric antrum (2, 3, 22). In addition, oral challenge of human volunteers (15, 19) or gnotobiotic piglets (11, 12) is accompanied by histologic evidence of gastritis and, in humans, by symptoms of dyspepsia. Virtually nothing is known regarding factors that allow either colonization of the stomach or induction of gastritis by *C. pylori*. Differences in pathogenicity between isolates have been suggested. One report correlated serotype of clinical isolates with severity of associated gastritis (4), but virulence factors were not addressed. A variety of virulence factors have been proposed. *C. pylori* is highly motile, suggesting that motility may facilitate colonization of gastric mucus (9). Furthermore, some strains of *C. pylori* produce a protein cytotoxin which causes vacuolation in intestinal epithelial cell monolayers in vitro (14). This toxin may contribute to gastric inflammation and erosion in vivo. The purpose of this report is to describe differences in virulence in motile, nonmotile, toxicogenic, and nontoxicogenic strains of *C. pylori*.

### MATERIALS AND METHODS

**Bacterial strains.** Six strains of *C. pylori* were used in this study. Three were human isolates, two (60190 and 26695) were obtained from A. D. Pearson, and one (Tx30a) was obtained from G. E. Buck. All three had been passaged in culture for at least 2 years, and the severity of gastritis associated with the original isolates is not known. Strain 59r, 1553r, and 1554r were recovered from piglets challenged with human strains. Strain 59r was recovered from a piglet challenged with 26695. The other two strains were from piglets challenged with a mixture of 26695 and 60190. All six strains were microaerophilic gram-negative pleomorphic rods identified as *C. pylori* on the basis of prominent urease, oxidase, and catalase activities. Broth cultures were established as previously described (18). Briefly, brucella broth supplemented with 10% fetal calf serum was inoculated with 10<sup>5</sup> CFU/ml. Flasks were incubated at 37°C in an atmosphere of 10% CO<sub>2</sub> on a rotary shaker at 150 rpm. GCHI agar supplemented with trimethoprim, vancomycin, amphotericin, and polymyxin B sulfate (Regional Media Laboratories,

Lenexa, Kans.) and incubated at 37°C in 10% CO<sub>2</sub> was used for agar cultivation.

**Motility.** Samples of broth cultures were examined for motility by light microscopy with a charged hemacytometer. For quantitation of motile colonies, broth cultures were diluted to approximately 40 CFU/ml in brucella broth containing 10% fetal calf serum and 0.5% agar which was melted and then cooled to 42°C. Samples were poured into petri dishes, allowed to cool to room temperature, and incubated at 37°C in 10% CO<sub>2</sub> for 4 days. Colonial morphology was evaluated by examination at a magnification of ×40 with phase-contrast illumination. Colony diameter was measured with an ocular micrometer. For macroscopic assessment of motility, broth cultures were streaked onto soft agar with a wire loop and incubated as described above.

**Cytotoxin.** Cytotoxin in culture supernatant was titrated as previously described (14). Briefly, culture supernatants from 48-h cultures were harvested by centrifugation and filtered through 0.22-μm filters. Supernatants were concentrated 10-fold by precipitation in saturated ammonium sulfate and suspended in phosphate-buffered saline. Serial twofold dilutions of 10-fold ammonium sulfate concentrates (0.1 ml) were added to monolayers of Intestine 407 cells (ATCC CCL6) and incubated for 24 to 48 h. Monolayers were examined for vacuolation. A strain was considered to produce cytotoxic activity if more than 50% of the cells were vacuolated.

**Transmission electron microscopy.** One milliliter of broth culture containing 10<sup>8</sup> to 10<sup>9</sup> bacteria per ml was washed twice in water and suspended in 5% Formalin. Portions were mixed with an equal volume of 1% phosphotungstic acid and 3% sucrose (pH 7.0), layered on 300-mesh Formvar carbon-coated grids (Ladd Research Industries, Burlington, Vt.), allowed to air dry, and examined with a Phillips 300 electron microscope.

**Endonuclease restriction.** Endonuclease digestion of total cellular DNA was performed by a modification of the method of Langenberg et al. (13) with 1 μg of *Hind*III per 200 ng of DNA. Briefly, DNA from 10 ml of broth culture was isolated, and restrictions were carried out overnight at 37°C. Fragments were separated in a 0.7% horizontal agarose gel run at 70 V for 5 to 7 h.

**Animals.** A total of 33 piglets from four litters was used in this study. Piglets were derived by Caesarian section and maintained in sterile isolation units as previously described (11). Gnotobiotic conditions were verified by bacterial cul-

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ture of the isolators and the gastric mucosa, which revealed no bacteria other than *C. pylori*. Piglets were randomly assigned into infection groups. Groups receiving different strains of bacteria were housed in separate units. Animals were challenged at 3 days of age.

**Challenge inocula.** Bacteria in 36- to 48-h broth cultures were used for animal inoculation. Cultures containing  $10^8$  CFU/ml were harvested by centrifugation, suspended in peptone water, and enumerated by a standard plate count technique and a direct-count technique by using a hemacytometer. Only cultures containing short curved rods without clumps were used for animal challenge. Groups of piglets were challenged with  $10^9$  to  $10^{10}$  CFU of the following strains per piglet: group 1 (five piglets), 26695 alone; group 2 (eight piglets), both 26695 and 60190; group 3 (five piglets), 60190 alone; group 4 (six piglets), Tx30a; and group 5 (nine piglets), peptone water alone.

**Sample collection.** Piglets were sacrificed at 20 or 45 days after challenge. Animals were deeply sedated with ketamine and xylazine, removed from the isolators, and given 500 IU of heparin intravenously. Piglets were positioned in dorsal recumbency and euthanatized with intracardiac Uthol (Bolter Co., Columbus, Ohio), and the stomachs were exteriorized through a ventral midline surgical incision. Transmural biopsies were taken from four anatomic areas of the stomach (cardia, fundus, antrum, and pylorus) and were streaked onto GCHI agar plates for bacterial reisolation. Piglets were then positioned in right lateral recumbency, the left body wall was removed, and the piglets were perfused with Trumps fixative (4% formaldehyde and 1% glutaraldehyde in 200 mM phosphate buffer [pH 6.5]) via the descending aorta by a modification of the technique of Helander (10). Perfusion was carried out at 120 to 180 mm Hg for 15 to 20 min. Following perfusion, the stomachs were opened along the greater curvature and examined, and gross lesions were noted. The tissue was then immersed in 20 volumes of fixative. Sections for histopathology were embedded in paraffin, cut in 5- $\mu$ m sections, and stained with hematoxylin and eosin and Warthin-Starry stains. For transmission electron microscopy, sections of mucosa were minced, postfixed in 1.3% osmium tetroxide, and embedded in Medcast epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and were examined with a Phillips 300 electron microscope.

**Histopathologic examination.** For quantitation of inflammation, hematoxylin-eosin-stained sections of glandular cardiac mucosa from all piglets were examined. Surface area of lymphoid follicles and total surface area of the lamina propria were measured by using a ZIDAS (Zeiss Interactive Digital Analysis System; Carl Zeiss, Inc., Thornwood, N.Y.) at a magnification of  $\times 40$ . For quantitation of epithelial vacuolation, sections of cardia from selected individuals were examined. Individuals chosen were those in each group with the most severe inflammatory lesions. In the absence of inflammation, individuals were randomly selected. The number of vacuolated cells in each section was counted, and the length of the superficial epithelium was measured by using a ZIDAS at a magnification of  $\times 40$ . Warthin-Starry-stained sections were examined for the presence of bacteria and scored as either positive or negative. Sections were scored without prior knowledge of challenge status.

## RESULTS

**Bacterial motility.** Motility was evaluated by microscopic examination of wet mounts and by plating in soft agar. When

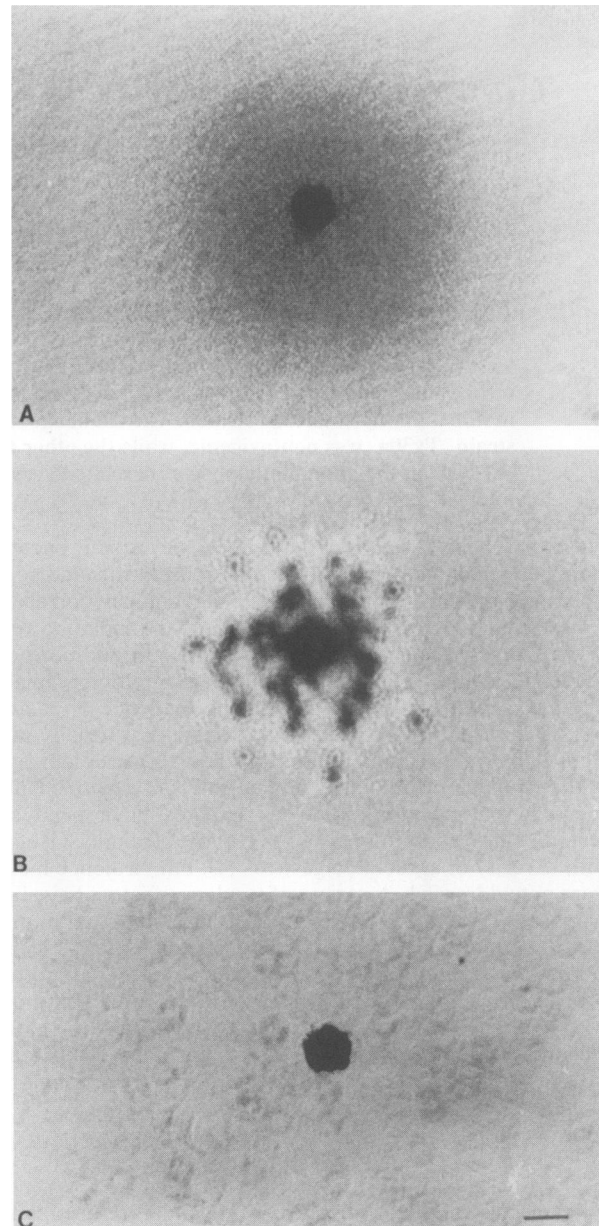


FIG. 1. Colonial morphology of three strains of *C. pylori*. (A) Strain 26695, typical spreading colony; (B) strain 60190, granular colony; (C) strain Tx30a, nonspreading colony. Bar = 0.25 mm.

plated in soft agar, the strains demonstrated several colonial morphologies (Fig. 1). These are described as spreading (Fig. 1A), granular (Fig. 1B), or dense (Fig. 1C). There was marked variation in the size of spreading colonies (0.5 to 2.5 mm in diameter). Granular colonies were approximately 0.5 to 1.0 mm and dense colonies were 0.25 mm in diameter. The colony types varied among the strains (Table 1). Of the challenge strains, strain 26695 produced the largest proportion of spreading colonies (28% of colonies) and Tx30a produced the least (10%). Strain 60190 produced mostly granular colonies. The three strains isolated from infected piglets were enriched in spreading colonies (greater than 90%). The presence of spreading with 26695 and the porcine isolates but not with 60190 or Tx30a could also be demon-

TABLE 1. Colonial morphology of *C. pylori*

Strain	Total no. of colonies counted	Colonial morphology (%) <sup>a</sup>		
		Spreading	Granular	Dense
26695	2,440	28 ± 16	0	72 ± 17
60190	3,317	4 ± 2	53 ± 34	44 ± 33
Tx30a	1,569	9 ± 0	0.5 ± 0.7	90.5 ± 0.7
59r	2,125	95 ± 4	2 ± 2	4 ± 2
1553r	277	99 <sup>b</sup>	0	1
1554r	383	98 <sup>b</sup>	0	2

<sup>a</sup> Values are means ± standard deviations of three trials.

<sup>b</sup> Values given represent a single trial.

strated by streaking the three strains in soft agar (Fig. 2). Strains 26695 and 59r showed wide spreading and poorly demarcated borders, while 60190 and Tx30a showed only narrow, sharply demarcated halos.

Wet mounts of all strains were inconsistently motile. Some broth cultures of 26695 and the porcine isolates showed rapid directional motility. Individual organisms traveled rapidly for several micrometers and then stopped, changed direction, or started to spin. In other cultures or at other times in the same culture, bacteria demonstrated only Brownian motion. Rapid directional motility was occasionally observed with 60190, which more often showed slow, undulant motility. Tx30a did not demonstrate motility. Thus,

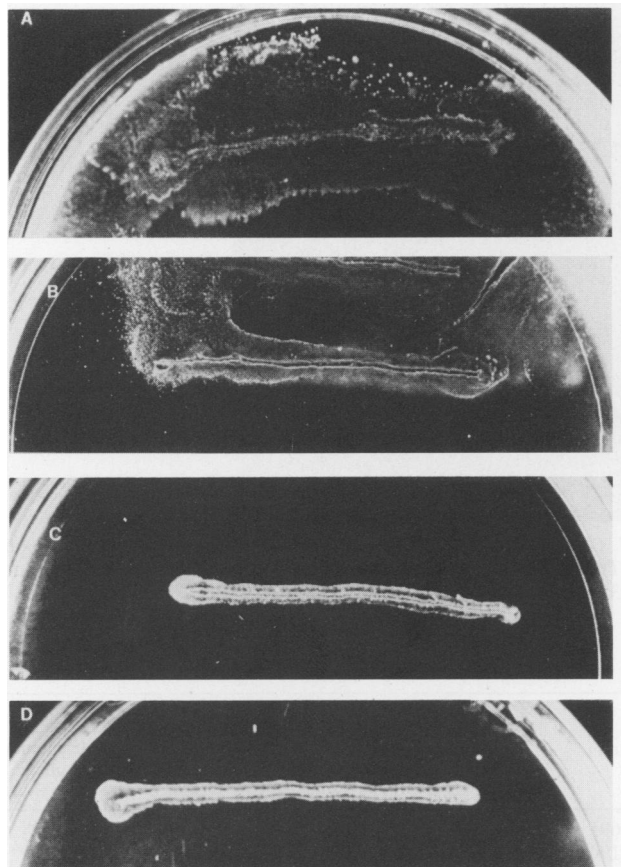


FIG. 2. Spreading in soft agar by four strains of *C. pylori*. (A and B) Strains 26695 (A) and 59r (B) spread widely, and borders are poorly demarcated. (C and D) Minimal spreading by strains 60190 (C) and Tx30a (D). Borders are sharply demarcated.

strain 26695 and the porcine isolates were highly motile, 60190 was weakly motile, and Tx30a showed minimal evidence of motility.

Transmission electron micrographs of the bacteria are shown in Fig. 3. Strains 26695 and 59r demonstrated the four to six polar flagella typical of *C. pylori*. The number of flagellated organisms varied with the individual inoculum, but flagellated organisms were always found. Flagellated Tx30a individuals were never found, and flagellated 60190 individuals were only occasionally present.

**Cytotoxin.** In addition to differences in motility, the strains differed in cytotoxin production. Table 2 shows different levels of toxin production by the strains. Strain 60190 had the highest titer of toxin activity in culture supernatant, 26695 and 59r had comparable titers, and Tx30a was nontoxic.

**Lesions.** Colonization of gnotobiotic pig stomachs with *C. pylori* was accompanied by gastritis characterized primarily by lymphocytic follicles and diffuse lymphocytic inflammation. Severity and distribution of inflammation varied, but in most instances follicles were large enough to be seen with a hand lens. Follicles were distributed mostly along the lesser curvature of the stomach, surrounding the cardia, and at the entrance to the diverticulum. Some follicles were present along the major gastric rugae. Follicles were rare at the greater curvature and absent at the pylorus. In addition to the previously described follicular gastritis, there was marked epithelial vacuolation in some piglets. Vacuoles were approximately 1 to 3  $\mu$ m and were clustered around the nucleus of the epithelial cell. They were more frequent near the cardia, but in individuals with the most vacuoles they were widely distributed. Transmission electron microscopy of vacuolated cells revealed large membrane-bound cavitations, sometimes containing flocculent debris (Fig. 4).

**Virulence.** Incidence of gastritis and infection rate were greatest in those piglets challenged with strain 26695 (Table 3). Of the four groups of piglets, those challenged with 26695 alone and those challenged with both 26695 and 60190 had infection rates of 100 and 75%, respectively. Those challenged with 60190 alone had an infection rate of only 40%. We were able to demonstrate bacteria in histologic sections of only one piglet challenged with Tx30a. Severity of gastritis paralleled the infection rate. Inflammation was most severe in piglets challenged either with 26695 alone or with both 26695 and 60190. Inflammation in piglets challenged with 60190 alone or Tx30a was less severe.

The incidence of epithelial vacuolation is shown in Table 4. Although vacuoles occurred in all groups, including uninfected controls, the mean number of vacuoles per millimeter of mucosa was greater in piglets challenged with 26695 than in the other groups.

Only strain 26695 was isolated from infected piglets. Recovery of *C. pylori* was successful only when the challenge inoculum included 26695 (Table 3). In addition, only 26695 was isolated from piglets challenged with both 26695 and 60190. Figure 5 shows *Hind*III endonuclease restriction patterns for the challenge strains and porcine isolates. Strains 1553r and 1554r, isolated from piglets challenged with both 26695 and 60190, had restriction patterns identical to those of 26695 but different from those of 60190 and the mixture of strains, indicating that 26695 and not 60190 was the virulent strain.

## DISCUSSION

Virtually nothing is known regarding virulence factors of *C. pylori*. Various mechanisms have been suggested by

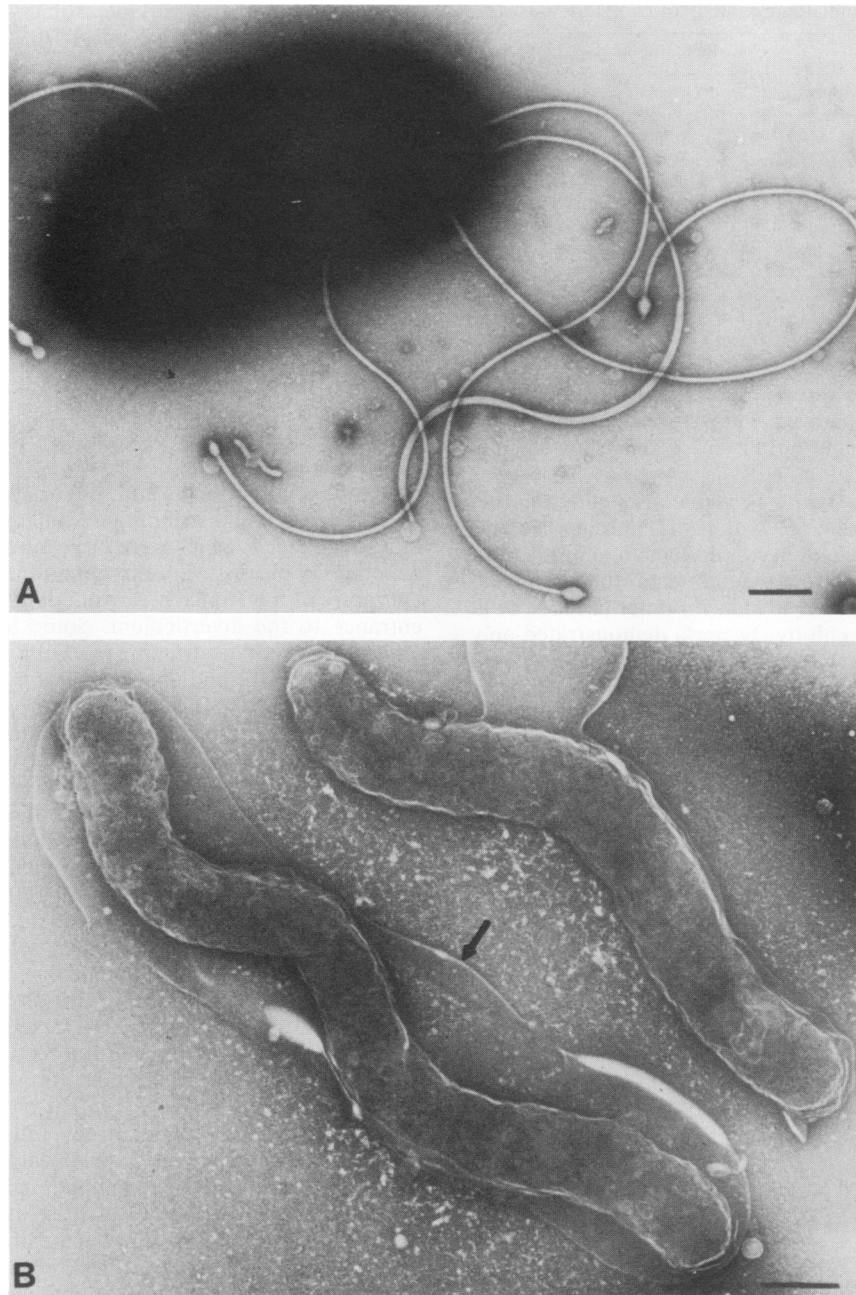


FIG. 3. Transmission electron micrographs of two strains of *C. pylori*. (A) Strain 26695. Bacterium demonstrates five sheathed polar flagella with terminal dilations typical of *C. pylori*. Bar = 20 nm. (B) Strain Tx30a. No flagella are present. Separation of the outer membrane (arrow) was commonly seen in electron micrographs of all strains. Bar = 35 nm.

which this organism may colonize the inhospitable environment of the stomach (3, 9), but lack of an animal model for infection has precluded testing of these proposals. We have used a porcine model of infection to compare the virulence of three strains which differ in motility and cytotoxin production.

Our data indicate that nonmotile or weakly motile and nontoxicogenic *C. pylori* are less virulent than a motile, toxicogenic strain. Infection rates with a nonmotile, nontoxicogenic strain (Tx30a) and a weakly motile strain (60190) were low. Furthermore, infection with these strains could be demonstrated only by Warthin-Starry stain and by evidence of

TABLE 2. Cytotoxin production by *C. pylori*

Strain	Cytotoxin titer <sup>a</sup> in:	
	Supernatant	Ammonium sulfate precipitate
26695	20	80
60190	40	160
Tx30a	0	0
59r	0	80

<sup>a</sup> Reciprocal of the last dilution which produced >50% vacuolation of Intestine 407 cells.

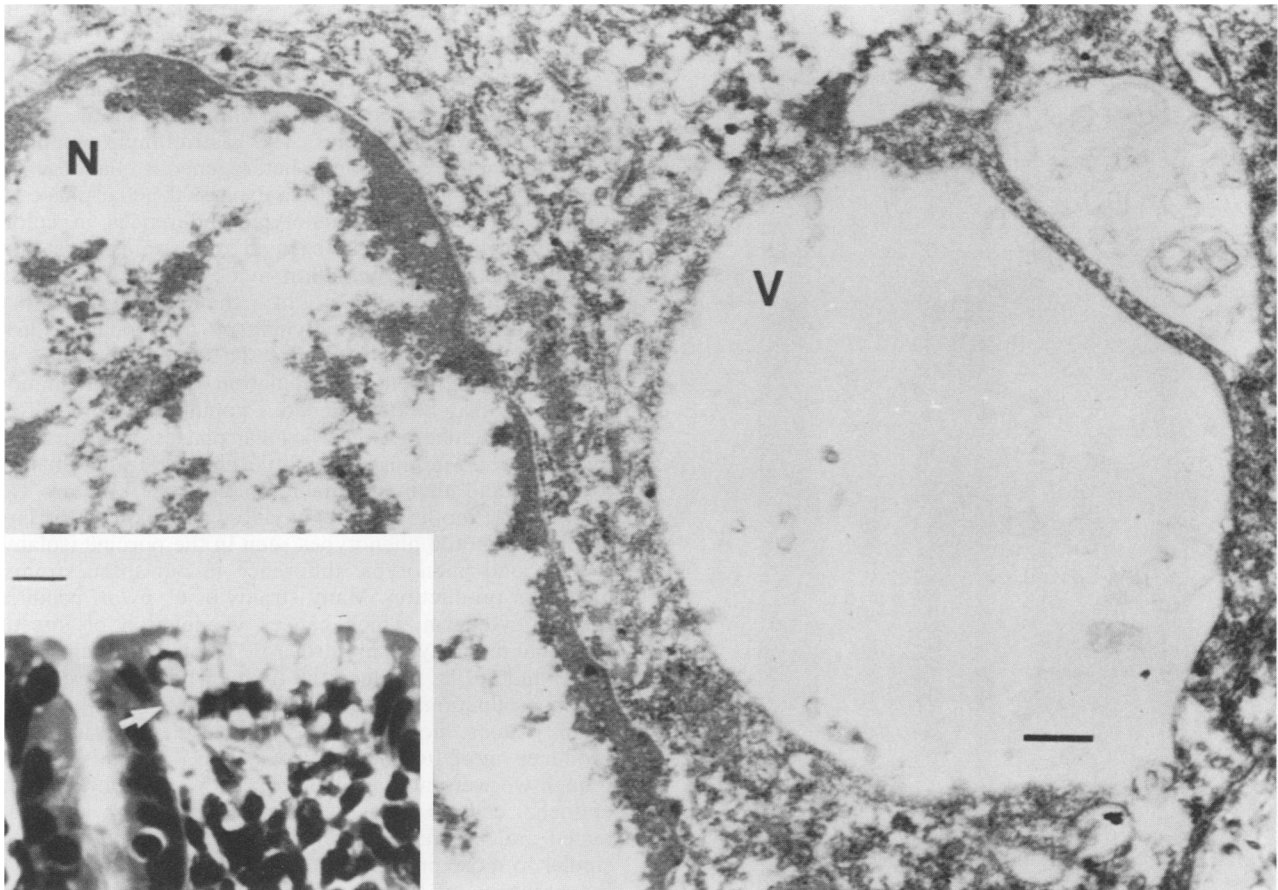


FIG. 4. Transmission electron micrograph of vacuolated epithelial cell. Two membrane-bound cytoplasmic vacuoles are present, both of which contain flocculent debris. N, Nucleus; V, vacuole. Bar = 35 nm. Inset: Light micrograph of hematoxylin-eosin-stained section of epithelial vacuoles (arrow). Bar = 20  $\mu$ m.

inflammation; bacteria were not isolated. In the single piglet challenged with Tx30a and demonstrating bacteria on the gastric mucosa, bacteria were rare (data not shown). In piglets challenged with the highly motile, toxigenic strain 26695, infection rates were high, approaching 100%. Furthermore, anatomic evidence of inflammation and epithelial vacuolation was greater in those groups challenged with 26695. Only 26695 was isolated from piglets challenged with

both 26695 and 60190, suggesting that of the two, 26695 is the virulent strain. Thus, three parameters of virulence indicate that 26695 is more pathogenic for gnotobiotic piglets than either 60190 or Tx30a. Infection rate and severity of inflammation were greater in piglets challenged with 26695 or the mixture of strains, and 26695 was the only strain recovered from piglets challenged with the mixture.

Differences in motility among the three strains may account for differences in pathogenicity. The most virulent strain, 26695, is the most motile. This strain demonstrates

TABLE 3. Results of infection of gnotobiotic piglets with various strains of *C. pylori*

Group	Bacterial strain(s)	Inflammation <sup>a</sup>	Colonization <sup>b</sup>		
			Culture	WS	Total (%) <sup>c</sup>
1	26695	0.388 $\pm$ 0.065	4/5	5/5	5/5 (100)
2	26695 and 60190	0.256 $\pm$ 0.086	5/8	4/8	6/8 (75)
3	60190	0.016 $\pm$ 0.011	0/5	2/5	2/5 (40)
4	Tx30a	0.021 $\pm$ 0.018	0/6	1/6	1/6 (17)
5	None	0.006 $\pm$ 0.003	0/9	0/9	0/9 (0)

<sup>a</sup> Surface area of lymphoid aggregates/total surface area of lamina propria,  $\pm$  standard error. Results for groups 1 and 2 are significantly different from those for controls ( $P < 0.05$ ).

<sup>b</sup> Number of piglets colonized/total number in group. Colonization was determined by detection of spiral bacteria by using Warthin-Starry (WS) stain or by culture of *C. pylori* from gastric mucosa.

<sup>c</sup> Total number colonized as demonstrated by either culture or Warthin-Starry stain.

TABLE 4. Gastric epithelial vacuolation in piglets infected with *C. pylori*

Group (n)	Bacterial strain(s)	No. of vacuolated cells/mm of gastric mucosa <sup>a</sup>
1 (11) <sup>b</sup>	26695 and 60190	12.8 $\pm$ 4.2 <sup>c</sup>
2 (3)	60190	6.2 $\pm$ 3.4
3 (3)	Tx30a	3.4 $\pm$ 0.7
4 (9)	None (control)	1.6 $\pm$ 0.7

<sup>a</sup> Mean  $\pm$  standard error.

<sup>b</sup> Piglets infected with 26695 and those infected with the mixture of strains were combined into a single group. Individuals included were those in each group with the most severe inflammation. All controls (group 4) were included.

<sup>c</sup> Significantly different from control value ( $P = 0.033$ ).

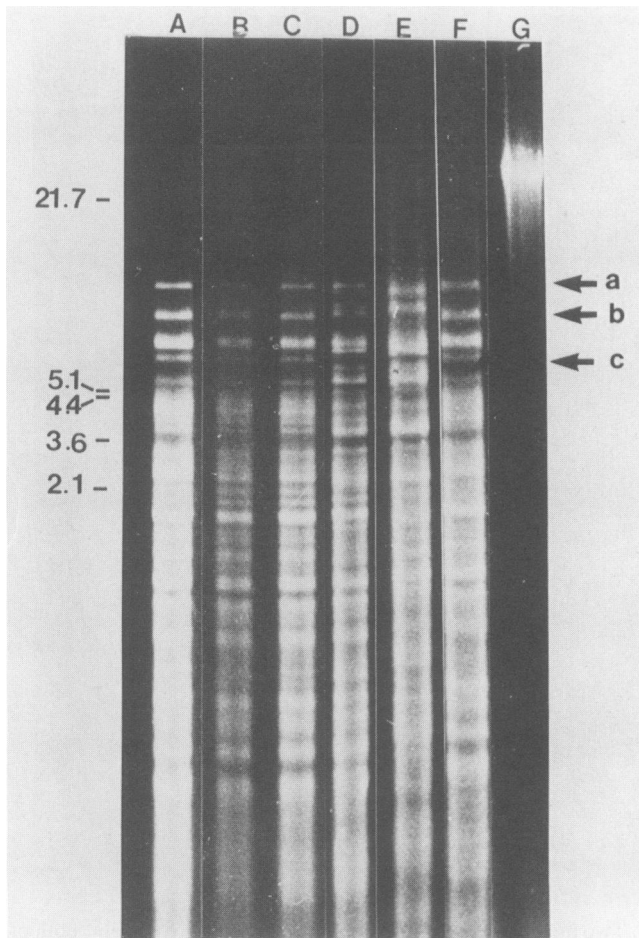


FIG. 5. Endonuclease restriction patterns of four strains of *C. pylori*. Lanes A and F, 26695; lane B, 1553r; lane C, 1554r; lane D, mixture of 26695 and 60190; lane E, 60190; lane G, uncut whole-cell DNA. Size markers (in kilobases) are at the left. Differences between the strains are most clearly seen by comparing the two largest fragments. The largest fragment (a) is slightly larger in 26695 and the porcine isolates than in 60190. Fragment b is slightly smaller in 26695 and the porcine isolates than in 60190. All four fragments are visible in the mix (lane D). In addition, fragment c is present in 60190 and the mix but not in 26695 or the porcine isolates.

the most consistent motility in wet mounts, produces the largest number of spreading colonies and the largest haloes on soft agar, and is consistently flagellated when examined by transmission electron microscopy. The less virulent strain, 60190, demonstrates weaker motility with smaller, granular haloes in soft agar and inconsistent motility in wet mounts. The nonpathogenic strain, Tx30a, is nonmotile. The correlation of virulence with motility can be accounted for in several ways (3, 9, 21). Motility is an established virulence factor for other bacterial pathogens. Nonmotile, aflagellate *Vibrio cholerae* strains are nonpathogenic (7, 23). In addition, nonmotile aflagellate and flagellated *Pseudomonas* spp. have a greatly diminished pathogenicity for wound infection (6, 17). Similarly, motility in *C. pylori* may facilitate penetration of gastric mucus and colonization (9). Alternatively, flagella may promote adherence between bacteria and host epithelium, thus facilitating colonization, as has been suggested for *Campylobacter jejuni* (16) and *V. cholerae* (1).

In addition to the correlation between motility and virulence of the challenge strains, gastric colonization appears to

favor motile forms. All three porcine isolates had more than 90% spreading colonies compared with less than 30% for the challenge strain (26695). Bacteria were not recovered from piglets challenged with Tx30a and 60190, and thus, motility could not be evaluated. This enrichment of motile bacteria could arise in several ways. The gastric environment may select for motile cells from a heterogeneous challenge inoculum. Many flagellated bacteria express flagellar phase variation (5, 8, 20). The phenotypic differences in colonial morphology on soft agar seen in our strains most likely reflect similar phase variation in *C. pylori*. If the gastric environment selects for motility, then the strain with the largest number of motile individuals in the inoculum (26695) would be most likely to colonize. The challenge strains may consist of a heterogeneous population of motile and immotile variants or may be homogeneous populations with differing rates of switching between flagellar phases. Growth in vivo may induce switching to a motile phase. Strains unable to switch would then be unable to colonize. In any case, selection for motile variants in vivo would explain the larger number of motile phenotypes seen in the porcine isolates.

A second phenotypic difference in our three strains is cytotoxin production. Many strains of *C. pylori* produce a protein cytotoxin which causes vacuolation of intestinal epithelial monolayers in vitro (14). The two virulent strains, 26695 and 60190, produce the toxin, while Tx30a does not. Toxin production correlates less well with virulence than motility does, however. The less virulent strain, 60190, produces more toxin than does 26695. Furthermore, although we were able to demonstrate vacuolation of gastric epithelial cells in vivo, vacuoles were present in both infected and uninfected individuals. These vacuoles appear similar to those produced in vitro by the cytotoxin (14), but the presence of these vacuoles in uninfected individuals suggests that they are not necessarily a direct result of infection. They may be attributable to normal epithelial turnover (enhanced by infection) or to inflammatory cell mediators. Finally, we were unable to demonstrate toxin activity in vivo either by direct titration of gastric juice for toxin activity or by demonstration of serologic response to toxin (data not shown). Although these data suggest that toxin is not active in piglets, it may be that our assays are insufficiently sensitive to identify low levels of toxin activity.

Thus, we have demonstrated that a virulent strain, 26695, is both toxigenic and motile; that a less virulent strain, 60190, is less motile and toxigenic; and that an avirulent strain, Tx30a, is nonmotile and nontoxigenic. It may be that both toxin production and motility are necessary for colonization of gnotobiotic pig stomachs. Further studies are necessary to clarify the relative importance of these factors.

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