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Evidence for Ethnic Tropism of *Helicobacter pylori*

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Helicobacter pylori **infection in humans is linked to gastritis, gastric and duodenal ulcers, and gastric cancer. Peptic ulcer disease, as distinct from chronic asymptomatic infection, is strongly associated with expression of bacterial virulence markers, including a major antigen, CagA, and the vacuolating cytotoxin VacA. We have previously described significant differences in colonization rates, independent of socioeconomic status, among ethnic groups in New Zealand. To evaluate relative risks for peptic ulcer disease, we examined the frequency of two virulence markers in *H. pylori* strains infecting these ethnic groups. Although these markers occurred significantly more frequently in strains isolated from Polynesians than in strains from Europeans, this frequency was not reflected in the incidence of peptic ulcer disease in the two groups. DNA fingerprinting of the urease gene showed that Polynesians are more frequently infected by a group of strains which are genetically distinct from those affecting European New Zealanders. Our data suggest that separate bacterial lineages may have evolved in parallel with race-specific specialization.**

Helicobacter pylori is a spiral, gram-negative, microaerophilic bacterium associated with chronic superficial gastritis and peptic ulcer disease of humans (5). In addition, epidemiological studies suggest a significant correlation between prevalence of *H. pylori* gastritis and gastric cancer in many countries, and individuals who have *H. pylori* infection have a three- to sixfold increased risk of gastric cancer (16, 19, 27).

Peptic ulcer disease due to *H. pylori* infection, as distinct from chronic asymptomatic infection, is strongly associated with expression of bacterial virulence markers, including a major antigen, CagA, and the vacuolating cytotoxin VacA (6, 41). Furthermore, recent evidence has shown an association between infection with a CagA-producing strain and the development of both atrophic gastritis (28) and adenocarcinoma of the stomach (7, 13). These properties of CagA-producing strains are probably due partly or wholly to the products of a multigene locus upstream of *cagA* (40).

The frequency of the CagA and VacA markers in strains infecting populations is therefore likely to be crucial in determining the disease patterns which result. The *vacA* gene is present in essentially all strains tested, but about 50% of clinical isolates produce inactive (or less-active) toxins due to alleles characterized by differences in the signal peptide and/or middle region of the gene (10). The *cagA* gene is present in about 60% of *H. pylori* strains in developed countries (6), and in the most recent analysis of 17 countries, seropositivity rates varied from a low of 33% in Iceland to 66% in Belgium (12). In previous studies, we showed a high seroprevalence of *H. pylori* in Maori and Pacific Islanders in New Zealand independent of age and socioeconomic status (21, 23). Colonization was consistent with the frequency of gastric cancer (23), which is significantly higher in Polynesians (Maori and Pacific Islanders) than in New Zealanders of European descent (39). It therefore seemed appropriate to survey the phenotypic properties of strains isolated from Polynesians. In this report, we document the elevated frequency of CagA-producing cytotoxic strains isolated from Polynesians and present data suggesting evolution of strains preferentially colonizing Polynesians.

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MATERIALS AND METHODS

Biopsy samples, bacterial strains, and culture conditions. Patients from whom antral biopsy samples were taken were attending the Gastroenterology Unit of the Middlemore Hospital, Otahuhu, Auckland, New Zealand, for a variety of gastric disorders. This hospital draws patients from an ethnically mixed but socioeconomically homogeneous population in South Auckland. Final diagnoses for the patients from which the 84 strains were isolated were duodenal ulcers (38%), normal (12.5%), gastritis (11%), gastric or duodenal erosions (9.5%), gastric ulcer (8%), esophagitis (8%), and other diagnoses (13%). All gastric biopsy samples were examined at the pathology department, Middlemore Hospital, by a single pathologist. *H. pylori* strains were cultured from biopsy samples on Columbia agar base plates supplemented with 5% horse blood under microaerophilic conditions as described previously (24). Frozen stocks of pure cultures were prepared and stored at −70°C.

Water extracts. Bacterial extracts containing vacuolating cytotoxin were prepared essentially as described before (41). Briefly, 3-day cultures were harvested in 1 ml of sterile distilled water, and the A_{600} was measured and adjusted to 2.0 with sterile distilled water. Surface protein removal and osmotic shock were accomplished by vortexing the cells at room temperature. Cells were removed by centrifugation at 13,000 × *g* for 20 s. The supernatant, referred to as water extract, was stored at −70°C.

Western blot (immunoblot) analysis of CagA and VacA production. Water extracts prepared as described above were separated in a 7.5% acrylamide gel containing sodium dodecyl sulfate (28a) in a minigel format (8- by 5-cm separating gel). Separated proteins were transferred from the gel to nitrocellulose paper by the methanol Tris-glycine system described by Towbin et al. (37). Electroblotting and subsequent steps were carried out by use of standard methodology (31) with a 1:10,000 dilution of VacA antibody and a 1:1,000 dilution of CagA antibody. Swine antirabbit antibody coupled to alkaline phosphatase (Sigma, St. Louis, Mo.) was used as a secondary antibody. The reactive bands were visualized by the method of Blake et al. (4) with 5-bromo-4-chloro-3-indolyl phosphate (Boehringer GmbH) as the alkaline phosphatase substrate and nitroblue tetrazolium (Sigma) as the color development reagent.

Vacuolization assays. An established human embryonic lung cell line (HEL 299; ATCC CCL 137) was employed for the vacuolization assay. Preliminary trials with culture supernatants of the known cytotoxic strain CCUG 17874 showed the toxin sensitivity of the lung cells to be comparable to that of HeLa cells (data not shown). Lung cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [pH 7.4]), 2 mM L-glutamine, 50 U of penicillin, and 50 μg of streptomycin in 25-cm^2 tissue culture flasks at 37°C (5% CO_2). Cells were trypsinized, resuspended in 5 ml of DMEM, seeded in a 96-well microtiter plate at a concentration of 10^4 cells per well in a final volume of 100 μl, and incubated for 24 h (11, 41). Water extracts were diluted 1:2 in DMEM. The human lung cells were washed once with phosphate-buffered saline (pH 7.2) just prior to toxin addition. Water extract (100 μl) was added to each well containing cells and incubated for 24 h (37°C, 5% CO_2). Vacuolization was assessed by light microscopy following fixation of cells with 2% formalin and

TABLE 1. Frequency of *H. pylori* virulence markers in strains from ethnic groups

Ethnic group[a] of subjects (*n*)	Frequency (%) of marker				
	CagA		VacA		CagA$^+$ cytotoxic strains[b] (%)
	Positive by immunoblot	PCR positive, immunoblot negative	Positive for cytotoxicity	Immunoblot positive, cytotoxicity negative	
European (48)	82	4	56	29	56
Polynesian (29)	96	3	89	10	89
Others (7)	85	14	85	14	71
Overall (84)	86	5	70	21	65

[a] The ethnic group of the subject was self-defined. The Polynesian group includes Maori and Pacific Islander (includes Tongan, Nuiean, Cook Islander, Tokelau, and Samoa). The group "Others" includes Indian, Chinese, Iraqi, South American, and Southeast Asian.
[b] CagA phenotype assayed by Western blotting with CagA-specific antiserum.

staining with crystal violet. Vacuolization of greater than 50% of cells in a well was defined as a positive result.

Preparation of template DNA. Crude chromosomal preparations (33) consistently yielded large quantities of PCR product and were prepared as follows. Cultures were harvested after 2 days of growth by scraping growth from the surface of the plate and resuspending the cells in 1 ml of prewarmed Luria broth. Cells were pelleted (13,000 × *g*, 20 s) and resuspended in 100 μl of TE buffer (10 mM Tris-Cl [pH 8.0], 1 mM EDTA). Then, 500 μl of GES (5 M guanidium thiocyanate, 100 mM EDTA [pH 8], 0.5% [wt/vol] Sarkosyl) and 2 μl of RNase (10 mg/ml) were added, and the suspension was mixed well by inversion until clear and incubated at room temperature for 10 min. Ice-cold 95% ethanol (1 ml) was added to precipitate the DNA. The DNA was pelleted by centrifugation (13,000 × *g*, 20 s), washed twice with 70% ethanol, and dried under vacuum. DNA was resuspended in 200 μl of TE buffer, and the concentration was adjusted to 1 ng/μl by spectrophotometric determination and dilution as necessary. Volumes of 1 μl were used in PCR amplifications.

PCR. PCR amplifications of the *ureA-ureB*, *cagA*, and *vacA* genes were performed with primers UREAB-1 (5′-AGGAGAATGAGATGA-3′) and UREAB-2 (5′-ACTTTATTGGCTGGT-3′) for the 2.4-kb *ureA-ureB* gene segment (20), *cagA* 1 (5′-AGTAAGGAGAAACAATGA-3′) and *cagA* 2 (5′-AATAAGC CTTAGAGTCTTTTTGGAAATC-3′) for the 1.35 kb-*cagA* gene segment, and *vacA* 1 (5′-GCTTCTCTTACCACCAATGC-3′) and *vacA* 2 (5′-TGTCAG GGTTGTTCACCATG-3′) for the 1.16-kb *vacA* gene segment (41). Amplifications were performed as described previously for the *ureA-ureB* (20), *vacA*, and *cagA* segments (41).

Genetic typing. Amplified DNA was ethanol precipitated, washed in 70% ethanol, and resuspended in sterile water. Restriction digestions were performed in a 25-μl reaction volume with 5 to 10 U of enzyme (either *Hae*III or *Mbo*I) and the buffer recommended by the enzyme supplier at 37°C for 2 h. Digested PCR products were separated by electrophoresis in a 6% acrylamide gel (60 V, 2.5 h). The restriction patterns produced were defined by molecular size determined by gel scanning and comparison to size markers (Life Technologies Inc.). Strains with identical patterns were assigned to the same group. Band sizes for each strain were entered into the Dendron program for matrix construction (35). A distance measure between individual strains was calculated as $(1 - S_{SM})$, with S_{SM} being a simple matching coefficient ranging from 0 to 1 (obtained by dividing the number of restriction fragments of identical size present in the patterns of both strains by the number of all restriction fragments present in the patterns of both strains) (9). A neighbor-joining tree (34) was then constructed from pairwise distances between the strains by use of PHYLIP (18).

RESULTS AND DISCUSSION

Phenotypic analysis of *H. pylori* isolates. To survey the *cagA* and *vacA* gene distribution, *H. pylori* was cultured from 84 patients, bacterial DNA was isolated, and the *cagA* and *vacA* genes were detected by PCR. To determine whether the *vacA* gene was expressed, cell extracts were tested by immunoblotting with specific antisera, and to distinguish nonvacuolating variants of VacA, cytotoxicity tests with lung cells were performed. These data (Table 1) show that strains likely to be ulcerogenic (expressing CagA and biologically active VacA) were significantly more frequently isolated from Polynesians than from Europeans (89 and 56%, respectively; $P < 0.01$, *z* test). Differences in the frequency of virulence markers of *H. pylori* strains in certain ethnic groups have also been reported in one study in the San Francisco Bay area (32), where African Americans were at a significantly higher risk of infection by strains producing active vacuolating cytotoxin and CagA than whites or Hispanics. The authors suggested either genetic predisposition of ethnic groups to infection with a particular phenotype or person-to-person transmission within relatively segregated populations as possible explanations, both of which we have also considered (see below).

VacA status. All strains which tested positive for vacuolating activity against the cell line produced an immunoreactive product detected in blots (data not shown). In a recent study, strains possessing an active cytotoxin indicated by the s1a signal sequence were associated with enhanced gastric inflammation and duodenal ulceration (3). Table 1 shows that significant numbers of strains produced a nonactive VacA protein, presumably harboring one of the particular alleles associated with this phenotype (2). Such strains were almost three times more frequently isolated from Europeans than from Polynesians. Only 56% of European-derived strains were cytotoxic compared to 89% of strains from Polynesians. Given the association of VacA with more severe disease, particularly duodenal ulceration (3), the frequency of duodenal ulceration would be expected to be higher in Polynesians. However, only 25% of Polynesian patients were diagnosed with duodenal ulceration compared to 46% of European patients. None of the European patients presented with gastric ulceration, compared to seven such cases among Polynesians, two of which were infected with noncytotoxic strains. The association of active VacA with severity of disease is therefore not borne out in this sample population.

CagA status. Relatively few strains (4 of 84) harbored a *cagA* gene that was either not expressed or failed to produce an immunologically reactive protein. Xiang and coworkers (41) reported 4 of 33 strains which had the *cagA* gene but appeared not to express it, at least 1 of which appeared to be due to a chromosomal rearrangement on the basis of an altered hybridization pattern. That study concurs with ours in showing that the majority of strains possessing the *cagA* gene express it.

The strong association of CagA-producing strains with duodenal ulceration was similar for the two major ethnic groups in this study. Of strains isolated from Europeans with duodenal ulceration, 83% were positive for CagA production and 86% were positive for Polynesian-derived strains. There were too few cases of gastric ulcers to make this comparison. Based on a lack of association between *cagA* and peptic ulcer disease in China, it has recently been suggested that CagA may be an important but insufficient factor in some disease processes in particular populations (27). It has also been suggested that CagA may merely be a marker for other virulence factors (6).

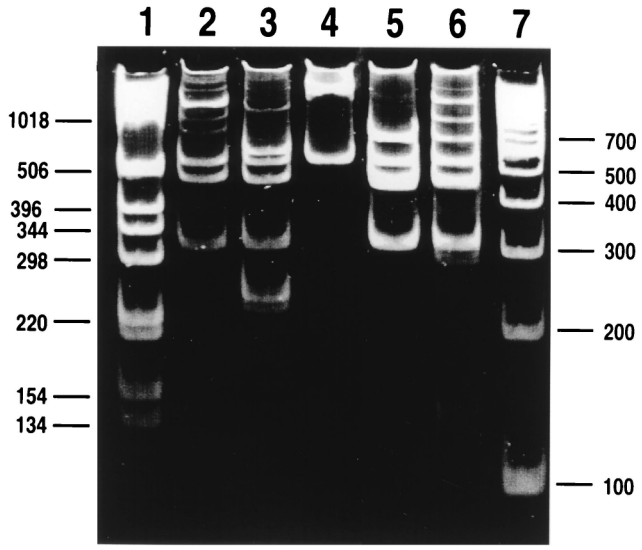

FIG. 1. RFLP analysis of the *ureA-ureB* region of *H. pylori*. A 2.4-kb region was amplified by PCR and digested with *Hae*III, and fragments were separated in a 6% acrylamide gel. Lanes: 1 and 7, size markers of the indicated numbers of base pairs; 2, strain 94 (cluster 1); 3, strain 83 (cluster 2); 4, strain 2 (cluster 3); 5, strain 5 (cluster 4); 6, CCUG 17874.

RFLP and genetic fingerprinting of *H. pylori* strains. To investigate the genetic relationships between strains colonizing ethnic groups, we chose a gene unrelated to *vacA* or *cagA* for fingerprinting the strain collection. PCR-based restriction fragment length polymorphism (RFLP) analysis has been successfully employed by others to differentiate isolates of *H. pylori* and to survey genetic diversity (1, 20, 22). We amplified a region encoding the urease A and B genes from the 84 strains and digested the PCR product with *Hae*III to generate patterns for typing (Fig. 1). Analysis of these patterns allowed division of the 84 strains into 29 distinct groups, which is comparable to the discrimination of 27 groups from 60 isolates in a previous study using identical methodology (1). Notwithstanding the difficulty of accurately comparing data in published figures, of the 27 groups we identified, only three seemed to correspond directly to the RFLP patterns of Akopyanz and coworkers (1), underlining the extraordinary genetic diversity of this organism (36).

A tree constructed from pairwise distances derived from the *Hae*III digestion patterns revealed that 82 of the 84 strains could be assigned to four major clusters (Fig. 2). Strains from clusters 1, 2, and 4 were significantly ($P < 0.001$, z test) more prevalent in Europeans or others than in Polynesians: they were isolated from 75% of such patients but from only 14% of Polynesians. Conversely, strains from cluster 3 were significantly more prevalent in Polynesians than in Europeans or others (86 versus 21%; $P < 0.001$, z test). We also tested for statistically significant ($P < 0.05$, z test) differences between clusters with respect to a variety of other host or strain properties, including diagnosis, gender, age, and antibiotic sensitivity of the strain, but none were found.

As expected from the elevated frequency of bacterial virulence determinants among Polynesians (Table 1), cluster 3, comprising predominantly isolates from Polynesians, contained predominantly CagA-producing cytotoxic strains (termed virulent strains). These results raised the possibility that the high frequency of virulence determinants among isolates from Polynesians might be merely a secondary effect of the association with Polynesians of a group of genetically similar strains which happen to frequently possess these determinants. This might indeed be the case. When we examined the frequency of VacA and CagA production in isolates from Polynesians, we found that only 3 of 5 isolates outside cluster 3 but 23 of 24 isolates in cluster 3 produced both of these virulence factors ($P < 0.05$, z test). The data suggest that these virulence factors may not be more important if *H. pylori* is to infect Polynesians than for infection of Europeans and others.

The existence of cluster 3 suggests that additional virulence may be required for successful infection of patients of Polynesian descent. If one considers only virulent strains in this study, such virulent cluster 3 strains were isolated from Polynesians almost eight times more frequently than virulent strains outside cluster 3 (23 virulent cluster 3 strains versus 3 virulent noncluster 3 strains; $P < 0.001$, z test). This is unlikely to be the result of transmission of cluster 3 strains due to social interaction among patients of Polynesian descent, since Polynesians in New Zealand fall into separate cultural groups, i.e., Maori and Pacific Islander. Demographic statistics on the frequency of households shared by Maori, Pacific Islanders, and Europeans show that both Maori and Pacific Islanders interact more frequently with Europeans than they do with each other. Maori share households 10 times more often with Europeans than with Pacific Islanders, and Pacific Islanders share households 1.7 times more often with Europeans than with Maori (data not shown [derived from Statistics New Zealand, 1991 Census coresidence data]). Even though the two cultural groups of Polynesian descent interact less frequently with each other than with Europeans in the general population, virulent cluster 3 strains are found with comparably high levels of prevalence in the 15 Pacific Island subjects and in the 14 Maori subjects surveyed (80 and 78%, respectively). Unfortunately, coresidence data were not available for the study population. If one accepts national patterns, it may be inferred that the elevated prevalence of virulent cluster 3 strains in Polynesian patients is best explained as the result of adaptation of cluster 3 strains to the Polynesian genetic background, indicating the existence of an additional virulence or colonization factor present in cluster 3 strains which facilitates the infection of patients of this genetic background.

It might be argued that it is the gene encoding urease, an essential virulence factor (14, 15, 38) on which the typing is based, which has evolved in separate lineages to facilitate ethno-specific colonization. The urease of *H. pylori* is universally produced by strains and has a central role in pathogenesis ascribed in part to neutralization of gastric acid, direct tissue damage, and indirect damage due to inflammatory response (reviewed recently by Mobley [29]). Assuming that urease performs a similar pathogenic function in all infected hosts, it would not be expected to specifically adapt in targeting ethnic groups; therefore, it seems more plausible that the division into European and non-European colonizing strains reported here is linked to some other race-specific recognition factors. An obvious target is the Lewis^b (Le^b) blood group antigen. The Le^a and Le^b antigens, typically found on erythrocytes, are also expressed on gastric epithelial cells, and the Le^b antigen has been shown to mediate *H. pylori* binding to human gastric mucosa (8, 17). It is well documented that Polynesians express the Le^b antigen at a low frequency and are generally weak secretors or nonsecretors (25, 26), while the Le(a−b+) erythrocyte phenotype occurs in approximately 75% of Europeans (30). Successful colonization of Polynesians by *H. pylori* should therefore require a strain capable of producing a novel bacterial adhesin, and it may be the existence of such a strain which the urease fingerprinting has detected. We are proceeding to

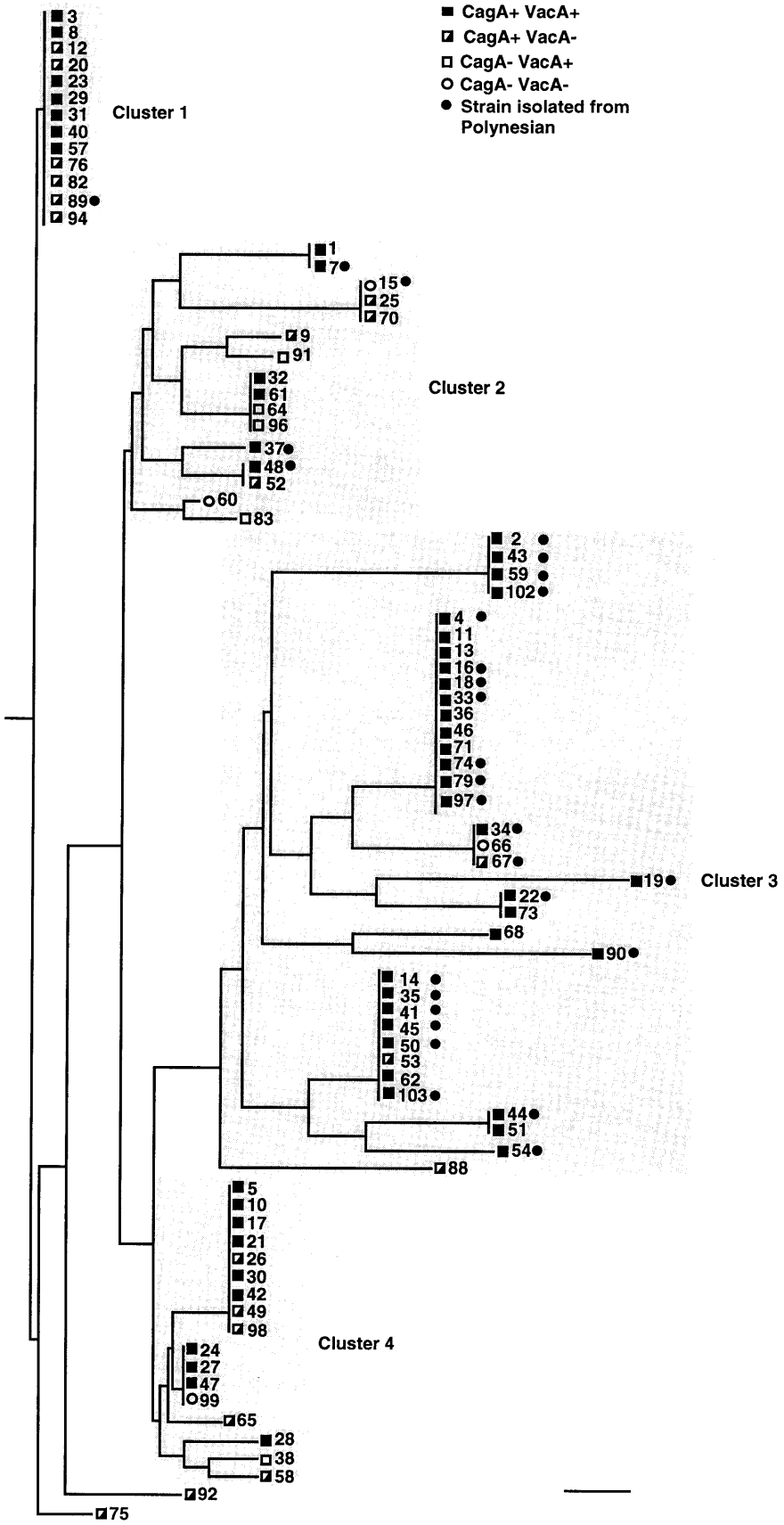

FIG. 2. Neighbor-joining tree constructed from pairwise distances between strains based on RFLP analysis of the *ureA-ureB* region of *H. pylori*. The bar in the figure represents a distance of $(1 - S_{SM}) = 0.1$. Symbols to the left of the strain numbers indicate phenotypes as described in Table 1. A closed circle to the right of a strain number indicates a strain isolated from a Polynesian patient.

type the patient population by Lewis blood group antigen and will investigate bacterial adhesion patterns to test this theory.

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